FOOD ADDITIVE PETITION

No. 3A4364

Aminoglycoside 3'-Phosphotransferase II (APH(3')II): Safety and Use in the Production of Genetically Engineered Plants

Calgene, Inc.

Environmental Assessment

[redacted]

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VOLUME II: ENVIRONMENTAL ASSESSMENT (EA)

Section A. Date: June 3, 1993

Section B. Name of Applicant/Petitioner: Calgene, Inc.

Section C. Address: 1920 Fifth Street, Davis, CA 95616

Section D. Description of the Proposed Action:

D.1 Request for Food Additive Regulation

Calgene, Inc. is requesting a food additive regulation from the Commissioner of Food and Drugs for aminoglycoside 3'-phosphotransferase II (APH(3')II) encoded by the *kan*^{r} gene, which is a selectable marker, used as a processing aid in the production of transgenic tomatoes, cotton and *Brassica napus* rapeseed.

D.2 Need for the Action

Calgene is developing the following proprietary, genetically engineered plant varieties and plant products:

- Tomatoes with improved agronomic and quality traits;
- Cotton with the patented BXN (bromoxynil resistant) gene conferring tolerance of the crop to the herbicide bromoxynil for improved weed control;
- Cotton resistant to specific insect pests such as the cotton bollworm and tobacco budworm; and
- Rapeseed varieties that will produce specialty edible and industrial oils. Edible rapeseed oil is produced from canola varieties in the U.S. and Canada.

The *kan*^{r} gene is contained in specifically identified vectors, where it functions as a selectable marker. These vectors will be used to make other constructs containing, in addition to the *kan*^{r} gene, the gene of interest. These other constructs, specific to each tomato, cotton and rapeseed product, will each be considered under a separate regulatory action based on the final product. The food additive regulation requested here is specifically for APH(3')II, the product of the *kan*^{r} gene, as contained in the designated vector constructs.

D.3 Production Location and Surrounding Environment

The *kan*^{r} gene will be produced and introduced into tomatoes, cotton and rapeseed at Calgene's principal research and development facility in Davis, CA. Calgene's principal research and development, executive and administrative offices at the Davis facility are located in four adjacent buildings totaling approximately 68,000 square feet. Calgene also owns a 23,000 square-foot greenhouse located on land near its Davis facility and leases another 51,750 square-feet of greenhouses in Galt, CA. These physical facilities are the primary sites where research is performed and plants containing the *kan*^{r} gene are developed and produced.

The elevation of the Davis facility is about 50 feet above mean sea level. It is bounded by Fifth Street on the north, Poleline Road on the east, Davis Waste Removal Service and recycling yard to the west, and the City of Davis water runoff pond on the south. This area of Davis is zoned for office and light industrial use. The Davis greenhouse is located on Second Street, a short distance to the east of Calgene's corporate offices and laboratories. It is bounded by hay storage, former trucking facilities, and Davis Waste Removal dumpster storage to the north; an access road, parking spaces and fields to the east; an unlined water runoff pond and fields to the west; and an abandoned truck weigh station, drainage ditch and fields to the south. The area beyond these facilities to the east, west and south are zoned for light industry and to the north for housing. Plant surveys are conducted periodically in areas adjacent to the greenhouse to comply with BL1 guidelines.

The greenhouse in Galt, CA, is located at 5401 East Jahant Road. The elevation of the site is about 75 feet AMSL. The greenhouse is bounded by livestock pastures and residences to the north, east and west and fallow ground to the south. The second greenhouse (21,000 sq. feet) is at 12675 Stockton Blvd. This site is surrounded by a 50 foot barren area and a 6 foot cyclone fence. It is bordered on the west by a frontage road and freeway and on the north , east and south by pasture land. Plant surveys are conducted periodically in areas adjacent to the greenhouse to comply with BL1 guidelines.

Stoneville Pedigreed Seed, a Calgene subsidiary in Leland, Mississippi, is Calgene's cotton breeding and commercial production facility. Calgene's rapeseed breeding and commercialization facility is located in Leesburg, GA. Tomato breeding is at the Davis, California facilities. Activities associated with breeding and production are identical for non-transformed and transformed tomatoes, cotton and rapeseed. Plant breeding stations consist primarily of offices and seed storage facilities. The stations are adjacent to or in the vicinity of field locations where breeding work is performed. Calgene has plant breeding stations for transgenic cotton and rapeseed at the following locations:

Stoneville Pedigreed Seed Company Southern Research Station Old Highway 61 Route 1, Box 39 Leland, MS 38756	Stoneville Pedigreed Seed Company Western Research Station Casablanca and Powerline Road P.O. Box 569 Maricopa, AZ 85239
Stoneville Pedigreed Seed Company Southeast Research Station 900 South 4th Street Building F Hartsville, SC 29550	Ameri-Can Pedigreed Seed Company 1190A U.S. Route 19 South Route 5, Box 70 Leesburg, GA 31762

D.4 Use Locations and Surrounding Environments

Seeds of tomato, cotton and rapeseed containing the *kan*^{r} gene produced by Calgene will be distributed in bulk to commercial growers, who will then plant, cultivate and harvest the respective crop using traditional agricultural methods. The harvest from the commercial sites may be used for end products (e.g., fresh or processed tomatoes, cotton oil, meal or fiber, and rapeseed oil or meal) or for seed increases for future production. Listed below are the states which have been identified as potential markets for seed sales of these three crops (USDA 1989):

- Tomato: Alabama, Arkansas, California, Colorado, Delaware, Florida, Georgia, Indiana, Louisiana, Maryland, Massachusetts, Michigan, New Jersey, New York, North Carolina, Ohio, Pennsylvania, South Carolina, Tennessee, Texas and Virginia.

- Cotton: Alabama, Arizona, Arkansas, California, Florida, Georgia, Kansas, Louisiana, Mississippi, Missouri, New Mexico, North Carolina, Oklahoma, South Carolina, Tennessee, Texas and Virginia.

- Rape: Alabama, Arkansas, California, Florida, Georgia, Idaho, Illinois, Indiana, Kentucky, Louisiana, Michigan, Mississippi, Missouri, Minnesota, Montana, North Carolina, North Dakota, Ohio, Oregon, South Carolina, Tennessee, Washington and Wisconsin.

The surrounding environments within and across the states listed above are highly variable, making it extremely difficult to assess environmental impact on a state-by-state basis. However, since we are looking at the worst case, our environmental risk assessment covers the environment in all states. Therefore, the potential environmental impact of growing and using

tomatoes, cotton and rape containing the *kan*^{r} gene will be evaluated from a generic perspective in this assessment.

D.5 Disposal Locations and Surrounding Environments

Because tomatoes, cotton and rapeseed products containing the *kan*^{r} gene and the gene product APH(3')II are intended strictly for human and animal use and/or consumption, the *kan*^{r} gene and gene product will be dispersed through use and not collected for disposal. However, we have developed and analyzed a scenario in which the entire crop is plowed under before harvest, and addressed the possible fate of the *kan*^{r} gene and gene products (Section G). This would represent the maximum amount of *kan*^{r} gene and gene product that could be introduced into the environment. Therefore, alternate means of introducing the *kan*^{r} gene and gene product into the environment through the direct disposal of the *kan*^{r} gene and gene product will not be considered in this EA as prescribed in the FDA publication, "Environmental Assessment Technical Assistance Document 2.00" (FDA 1987).

Section E. Identification of the Chemical Substance that is the Subject of the Proposed Action

Kanamycin-resistant genes occur naturally in the environment as evidenced by Henschke and Schmidt (1989). Also, Van Elsas and Pereira (1986) found background levels of kanamycin-resistant *Bacillus* ranging from 3300 to 30,000 CFUs per gram of soil in several different Brazilian soils. In this same study, kanamycin-resistant *Bacillus* populations were observed to comprise from 1 to 3 percent of the total *Bacillus* population within the different soil types examined. Therefore, although kanamycin-resistant bacteria represent a small percentage of the total soil microflora, they are present at detectable background levels. These background levels are also important in evaluating the environmental impact of the *kan*^{r} gene, as will be discussed in Section G.1.2.1.

The proposed use of the *kan*^{r} gene is to serve as a "selectable marker" in genetically engineered plants. The *kan*^{r} gene has a history of use in plants as a selectable marker (Budar *et al.* 1986, Fillatti *et al.* 1987, Catlin *et al.* 1988, Deroles and Gardner 1988, and Umbeck *et al.* 1989). It is also the most widely used selectable marker in mammalian cells (Sambrook *et al.* 1989), and has been most recently used in human gene therapy (See Section H.1.). Selectable markers are important in the genetic engineering of plants because transformation of plant cells by *Agrobacterium* is an inefficient process. In general, only a small minority of the plant cells exposed to DNA will successfully integrate and express that DNA. In order to provide a selection method for those plant cells which have been transformed, a "selectable marker" is incorporated into the transforming DNA. This selectable marker is a gene that confers a new phenotype to the recipient. The most widely used selectable markers in plants are genes that confer resistance to antibiotics. Following transformation with a gene construct containing an antibiotic resistance gene, plant cells are exposed to the appropriate antibiotic. Only those which are transformed survive. Incorporation of the *kan*^{r} gene into genetically engineered plants thus provides a method for selecting successfully transformed individuals.

Disarmed *A. tumefaciens* is the vector system of choice for delivery of genes into crop plants. This vector system has been extensively studied and is widely used for genetically engineering plants.

E.1 *kan*^{r} Gene

The *kan*^{r} gene used in genetically engineered plants developed by Calgene encodes an enzyme that confers resistance to the antibiotics neomycin, kanamycin and geneticin (G418). Neomycin and kanamycin are used therapeutically, while G418 is only used for *in vitro* experimentation. This *kan*^{r} gene, originally isolated from Transposon Tn*5*, encodes the enzyme aminoglycoside 3'-phosphotransferase II (APH(3')II).

[redacted]

The *kan*^{r} gene is commonly referred to as a neomycin resistance gene or kanamycin resistance gene, abbreviated *neo*^{r} or *kan*^{r}. The specific *kan*^{r} gene that is the subject of this assessment should not, however, be confused with other neomycin and kanamycin resistance genes that are similarly abbreviated but encode different enzymes (e.g., the *neo*^{r} gene originally isolated from transposon Tn*601* that encodes a type I aminoglycoside phosphotransferase enzyme). There are, in fact, a number of other naturally-occurring bacterial enzymes that inactivate kanamycin and neomycin, including acetyltransferases and nucleotidyltransferases, as well as phosphotransferases (Davies 1986, Davies and Smith 1978).

The complete nucleotide sequence for the *kan*^{r} gene has been published (Beck *et al.* 1982). Appendix EA-1 provides the nucleotide sequence for the *kan*^{r} gene constructs used in the production of genetically engineered crop plants by Calgene. There are seven constructs containing the *kan*^{r} gene (McBride and Summerfelt 1990), three of which have the *kan*^{r} gene spliced to mannopine synthase (*mas*) regulatory signals (5' promoter and 3' terminator sequences) and four which have the *kan*^{r} gene spliced to a CaMV 35S RNA promoter and *tml* terminator. Constructs pCGN1547, pCGN1548 and pCGN1549 have the *mas* regulatory signals and contain the same basic DNA sequence but differ from one another only in orientation of certain DNA fragments (McBride and Summerfelt 1990). Constructs pCGN1557, pCGN1558, pCGN1559 and pCGN1578 have the CaMV 35S/*tml* regulatory signals and also differ from one another only in orientation of certain DNA fragments (McBride and Summerfelt 1990). All the constructs contain a *lac* Z' locus (encodes *lac* alpha peptide), which contains a polylinker region from pUC18. The constructs are used for plant transformation only with a target gene inserted in the *lac* Z' locus, so no intact *lac* alpha peptide is encoded.

Specific gene sequences used to produce the FLAVR SAVR tomato are summarized in Table E.1-1 (Redenbaugh et al. 1992).

[redacted]

Table E.1-1. Gene Sequences in FLAVR SAVR Tomato

Gene	Sequence	Reference	Reference Sequence[3]
Left border (LB)	8-590	Barker 1983	626-1208
mas 5'	599-1277	Barker 1983	20806-20128
kan^r	1295-2276	Beck 1982	1539-2520
mas 3'	2283-3053	Barker 1983	19243-18473
Lac Z'[1]	3066-3342	Yanisch-Perron 1983	5999-6275
Lac Z'	3343-3354	"	6267-6256
Double 35S (d35S)[2]	3355-4568	Gardner 1981	6493-7342, 7069-7434
FLAVR SAVR™ gene	4585-6199	Sheehy et al. 1987	1617-2
Lac Z'	6200-6212	Yanisch-Perron 1983	6237-6248
tml 3'	6213-7358	Barker 1983	11209-10065
Lac Z'	7359-7532	Yanisch-Perron 1983	6276-6449
Right Border (RB)	7533-7817	Barker 1983	13992-14276
Overdrive T-Strand	7636-7659	Peralta 1986	--

1 Lac Z' is split at the insertion site of d35S and FLAVR SAVR genes and is consequently inactivated. Other Lac Z ' polylinker sequences are derived from cloning processes.

2 CaMV 35S = cauliflower mosaic virus 35S. The double represents a duplicated region in the promoter.

3 The reference sequences provide the precise nucleotide sequences for pCGN1436. Small differences may occur for different constructs and are due to cloning processes which regenerate sequences homologous to the original source of DNA or are sequences lost due to cloning processes such as klenow fill-in reactions which blunt the DNA and do not contain overlaps that would regenerate the restriction site sequences.

Constructs made from the seven vectors have been used for transformation of tomato, cotton and rapeseed and are stably integrated into the plant genome as shown by segregation analysis (Tables E.1-2 and E.1-3) and Southern analyses for (Sanders et al. 1992, Appendices EA-2 and EA-3).

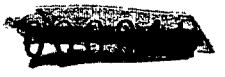


Table E.1-2. Analysis of Ripe Tomato Fruit Samples for APH(3')II Levels.

Transformant	Segregation Ratio	Estimated Functional *kan*^{r} Genes	ng APH(3')II per gm fr wt	% Total Protein
35S-*kan*				
2905-10	3:1	1	175	0.004
2905-8	15:1	2	35	0.0007
2905-2	3:1	1	140	0.003
2905-18	15:1	2	350	0.007
mas-kan				
7B-66	3:1	1	175	0.004
7b-107	3:1	1	175	0.004
28B-403	3:1	1	175	0.004
28B-404	3:1	1	175	0.004
7B-132	3:1	1	175	0.004

Table E.1-3. Nine Example BXN Cotton Events, Their Segregation Ratios Based on Buctril Resistance, and Levels of Nitrilase and APH(3')II in Leaves. A chi-square value of <3.84 means that there is a 95% probability that the true value is the predicted 3:1 segregation ratio.

Event #	Total:Susceptible T_2 Plants	Chi-Square Fit for 3:1 Ratio	% Nitrilase Protein in Leaves of T_3 Plants	% APH(3')II Protein in Leaves of T_3 Plants
10103	262:65	0.005	< 0.002	< 0.008
10109	173:42	0.48	< 0.002	< 0.008
10206	211:50	0.191	< 0.002	< 0.008
10208	241:51	1.893	< 0.002	< 0.008
10209	271:78	0.68	< 0.002	< 0.008
10211	241:53	1.163	< 0.002	< 0.008
10215	244:51	2.186	< 0.002	< 0.008
10222	240:52	1.422	< 0.002	< 0.008
10224	245:55	0.850	< 0.002	< 0.008

For the production of transgenic crop plants using the seven constructs described above, product specifications that will be maintained in the final product have been defined. No transgenic plant material with more than 10 copies of the *kan*^{r} gene per transformed somatic cell will be distributed for commercial use. Specific quality assurance (QA) and quality control (QC) procedures will be implemented to ensure that the defined product specifications are maintained. QA procedures as described in Appendix EA-4

will be used to measure the number of copies of the *kan*^{r} gene. For all calculations in the remainder of this assessment, we have used the conservative, upper-limit assumption that all transgenic crop plants released contain 10 copies of the *kan*^{r} gene per transformed somatic cell.

E.2 *kan*^{r} Gene Product: Aminoglycoside 3'-Phosphotransferase II (APH(3')II)

The specific form of neomycin phosphotransferase encoded by the *kan*^{r} gene is aminoglycoside 3'-phosphotransferase II (APH(3')II) (Beck *et al.* 1982). This enzyme catalyzes an ATP-dependent phosphorylation of the 3'-hydroxyl group on the aminohexose moiety of several aminoglycoside antibiotics. Phosphorylation of the antibiotics interferes with their uptake and ability to bind to cellular ribosomes, thereby rendering the cells resistant to the antibiotics.

The complete nucleotide sequence for the *kan*^{r} gene which encodes APH(3')II has been published (Beck *et al.* 1982). The nucleotide sequence of the *kan*^{r} gene used in the seven constructs is given in Appendix EA-1. The amino acid composition is shown in Appendix EA-5. APH(3')II contains 264 amino acids and has a calculated molecular weight of 29,053. Levels of APH(3')II were determined in FLAVR SAVR tomato fruit, in cotton leaves, seed and meal, and in rapeseed seed using a rabbit polyclonal antibody to develop an immunoblot (Tables E.1-2 and E.1-3; Appendix EA-6).

The bacterial promoter region associated with the wildtype gene from Tn*5* encoding APH(3')II, as defined by Beck et al. comprises the nucleotide sequence from position 1401 to position 1458 (Beck 1982). The *kan*^{r} gene sequence isolated from Tn*5* and used in the construction of pCGN1436 comprises the nucleotide sequence from position 1539 to position 2520 of the Beck *et al.* sequence (Table E.1-1). Therefore, the original bacterial promoter associated with the wildtype gene from Tn*5* encoding APH(3')II has been eliminated from the *kan*^{r} gene (and thereby from the transferred segment, i.e., the T-DNA) and could not possibly direct expression of the APH (3')II protein.

APH(3')II levels. APH(3')II levels were determined using Western analysis (see Appendix EA-6) and were significantly lower than 0.1% of the total protein in all cases. As an example, the APH(3')II signal from 600 µg of BXN cottonseed protein, loaded in one lane, had a weaker Western analysis signal than that seen in the lane containing 16 ng pure APH(3')II. Therefore, the concentration of APH(3')II was less than 0.003% of the total seed protein. Assuming seed protein is approximately 22% of seed weight (Ensminger et al. 1990), the maximum amount of APH(3')II in 1 gram of unprocessed cottonseed is

= (0.22 g protein/g seed)(0.003% APH(3')II)
= 6.6×10^{-6} g APH(3')II/g cottonseed
= 6.6 µg APH(3')II/g cottonseed

Cottonseed meal was also subjected to Western blot analysis. The APH(3')II signal from 600 µg of meal protein was less than the 8 ng purified APH(3')II signal in the western blots. Therefore, the concentration of APH(3')II was less than 0.0014% of the total meal protein. Assuming meal is approximately 41% protein (Ensminger et al. 1990), the maximum amount of APH(3')II in 1 gram of processed cottonseed meal is

= (0.41 g protein/g meal)(0.0014% APH(3')II)
= 5.8×10^{-6} g APH(3')II/g cottonseed meal
= 5.8 µg APH(3')II/g cottonseed meal

Based on these measurements, the levels of APH(3')II was less than 0.003% of the protein in unprocessed cottonseed and less that 0.0014% of the protein in processed cotton meal.

Similarly, levels of APH(3')II were measured in ripe tomato fruit (Table E.1-2) and in seed and processed meal of High Stearate rapeseed. The APH(3')II signals from 1200 µg of rapeseed protein and rapeseed leaves were less than the 10 ng signal of the purified APH(3')II in the western blots and the signal for 1200 µg of processed meal protein could not be detected (level of detection was 1 ng). Therefore, the levels of APH(3')II in High Stearate rapeseed whole seed and leaves are less than 0.0008% of the protein and less than 0.00008% of protein in the processed meal.

An upper quality control limit has been established for the maximum amount of APH(3')II that will be produced within a transgenic crop plant. No transformed plant material producing more than 0.1% of its total protein as APH(3')II will be distributed for commercial use. Specific QA/QC procedures will be implemented to ensure that this upper quality control limit for APH(3')II is maintained. QA procedures as described in Appendix EA-2 will be used to measure the level of APH(3')II. This procedure was previously included in Volume 1 (Food Additive Petition No. 3A4364) for tomato. Similar methods will be applied to cotton and rapeseed. For all calculations in the remainder of this assessment, we have used the conservative, upper-limit assumption that all transgenic crop plants released produce 0.1% of their total protein as APH(3')II. Results of Western blot experiments performed by Calgene demonstrate that APH(3')II levels in representative samples of ripe tomato fruit, cotton and rapeseed are less than 0.1% of the total protein (Section E.1 above, Appendices EA-2 and EA-3).

E.3 Additional Protein Coding Regions

The following information is provided to address the possibility that extraneous genes from *Agrobacterium tumefaciens, A. rhizogenes* and *Escherichia coli* K-12 may have been cloned into the region of the Ti plasmid transferred to plants. Information on tomato is provided.

FLAVR SAVR tomato. In addition to the DNA sequences encoding the APH(3') II protein and the reverse oriented (antisense) *Lycopersicon esculentum* polygalacturonase encoding DNA sequences, the following truncated protein coding regions are present between the 25 bp direct repeat sequences delineating the T-DNA region in pCGN1436.

- Within the DNA segment comprising the *mas* 5' gene, the promoter (5') and first 194 bp coding region of gene 25 from *A. tumefaciens* are present. This 194 bp portion represents less than 15% of the full length protein coding region. The gene 25 sequences in the *mas* 5' gene region, therefore, represent an extensive deletion mutation in gene 25. Within the DNA segment comprising the *mas* 3' gene, 95 bp of the 3' end of the *mas* gene coding region is also present.

- Within the DNA segment comprising the *kan*^{r} gene, the 5' portion of the *ble* gene from Tn*5* is present. The wildtype *ble* gene encodes resistance to bleomycin, a class of glycopeptide antibiotics produced by *Streptomyces verticillus* (Genilloud 1984). Only 154 bp of the 378 bp of the *ble* gene open reading frame (ORF) (Mazodier 1985) are present in the *kan*^{r} gene segment. No bacterial promoter is associated with the truncated *ble* gene as it is part of a bacterial operon the initial ORF of which is the *kan*^{r} (*neo*) gene (Genilloud 1984; Mazodier 1985). The bacterial promoter associated with the *kan*^{r} gene is no longer present in pCGN1436. Operons (as defined for bacteria) have not been identified in plants; therefore, the truncated *ble* gene should not be expressed in transformed plants.

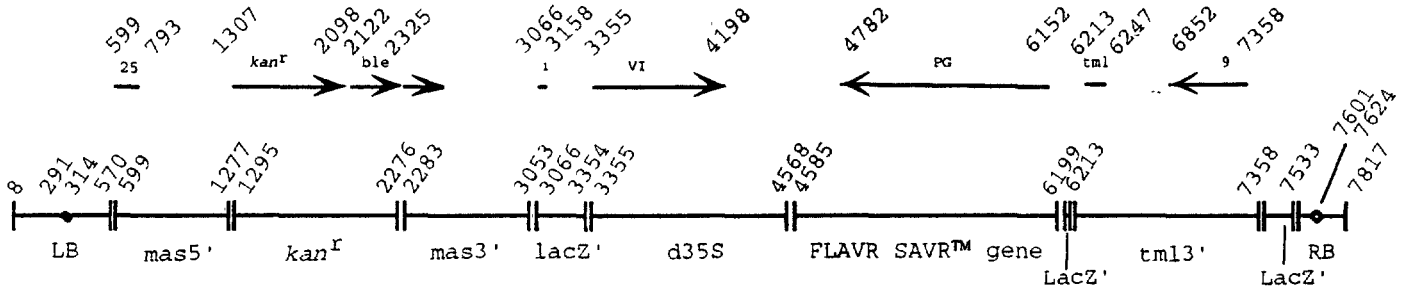
- Within the DNA segment comprising the *Lac* Z' gene region, the 3' portion of the *lac i* gene from *E. coli* is present. The wildtype *lac i* gene encodes the *Lac* repressor protein which is involved in regulation of the *lac* operon. Only 92 bp of the 1083 bp *lac i* ORF (Farabaugh 1978) are present in the *Lac* Z' gene segment.

- Within the DNA segment comprising the double 35S gene, the 3' portion of the cauliflower mosaic virus (CaMV) gene VI is present. As discussed by Zijlstra (Zijlstra and Hohn 1992), CaMV ORF VI protein is probably multifunctional. It is a major component of the inclusion bodies associated with the CaMV infection and may be involved in alteration of the cell's protein translation machinery. Only 843 bp of the 1562 bp comprising the ORF VI are present in pCGN1436. As such, the 843 bp

sequence represents a large deletion mutation of ORF VI, one lacking any eukaryotic regulatory sequences required for expression, and is not expected to be expressed. Zijlstra and Hohn (1992) also note that transformed *Arabidopsis* (and *Nicotiana*, by way of reference) plants expressing ORF VI were difficult to obtain and those that did regenerate were phenotypically abnormal. The complete lack of such severe abnormalities in tomato plants transformed with pCGN1436 further supports the conclusion that no expression occurs from this truncated ORF VI gene.

- Within the DNA segment comprising the *tml* 3' gene, the 3' portion of the ORF of the *tml* gene is present as is the 3' portion of the *A. tumefaciens* gene 9 (Barker 1983). Only 34 bp of the 1504 bp *tml* gene ORF is present and therefore this gene region is not expected to be expressed. Some 506 bp of the 576 bp that comprise gene 9 are present in pCGN1436; however, given the fact that the gene 9 promoter is not present on the cloned segment of the *tml* 3' gene region, the truncated gene 9 ORF should not be expressed in transformed plants.

Each of these truncated protein coding regions, along with the full length *kan*^{r} and polygalacturonase protein coding regions, are depicted on the map of the T-DNA region from pCGN1436 shown below.



E.4 Additives and Impurities

The *kan*^{r} gene constructs discussed in Section E.1. contain the nucleotide sequences shown in Appendix EA-1. These nucleotide sequences encode the *kan*^{r} gene product, APH(3')II, which has the amino acid sequence shown in Appendix EA-1 and the amino acid composition shown in Appendix EA-5. The *kan*^{r} gene constructs shown in Appendix EA-1 are the only coding sequences under consideration for this assessment. The *kan*^{r} gene contained in the constructs only encodes APH(3')II and does not code for any other full-length gene products. As discussed previously, the *lac Z'* locus will always be interrupted by a target gene and thus inactivated. Therefore, no non-

naturally occurring substances or impurities (e.g., non-naturally occurring residual materials, or byproducts of culture or processing) not specifically encoded by the inserted *kan*^{r} gene are expected to be produced.

[redacted]

Section F. Introduction of Substances into the Environment

Under contained conditions for the development of genetically engineered plants in laboratories and greenhouses, control of emissions, discharges and wastes resulting from production of tomatoes, cotton and rapeseed, containing the *kan*^{r} gene, are in compliance with current applicable Federal (i.e., National Institutes of Health, U.S. Environmental Protection Agency and U.S. Department of Agriculture), State (i.e., California and North Carolina) and local regulations and guidelines for the *kan*^{r} gene and plant material containing the *kan*^{r} gene. All chemicals used in the production of the *kan*^{r} containing plants are emitted, discharged or disposed of as waste only in accordance with permits.

After leaving the greenhouse and prior to commercialization tomato, cotton, and rapeseed plants will be field tested under provisions of the U.S. Department of Agriculture Animal and Plant Health Inspection Service (USDA APHIS), 7 CFR 340. Under either their permitting process or notification procedures, tomato, cotton and rapeseed plants containing the *kan*^{r} gene will be grown under conditions of containment specified by the USDA.

For commercial growing, all emissions, discharges and waste associated with the growing of cotton, tomato and rapeseed plants containing the *kan*^{r} gene will be identical to the emissions, discharges and waste from the presently grown non-transgenic crops.

This section of the EA includes a discussion of compliance with applicable regulations.

F.1 Overview of the Production of Transgenic Plants

The plant genetic engineering process can generally be divided into five major phases: (1) identification and isolation of genes and gene promoters (sequences of DNA that initiate gene expression), (2) transfer and integration of a gene or genes into the chromosomes of the recipient cell (i.e., transformation), (3) selection and regeneration of the transformed cells into whole plants using cell culture methods, (4) testing to confirm that the gene or genes transferred are correctly expressed in whole plants or selected plant tissues, and (5) genetic analysis to determine that successive generations consistently inherit and express the desired trait.

Once the five major phases are completed, field testing of the genetically engineered plants is done under contained conditions to determine agronomic and horticultural traits, yield, and usefulness for commercial production. Data from these field trials is used by the plant breeder to select superior lines for the next cycle of breeding and evaluation. During the field trialing process, the USDA APHIS is petitioned to determine that the

genetically engineered plants do not pose a plant pest risk and therefore are no longer regulated under 7 CFR 340. Finally, uncontained production of the plants begins for commercialization.

F.2 Laboratory, Greenhouse and Field Containment

This section of the EA discusses the safety of laboratory and greenhouse facilities and field trial sites where the *kan*^{r} gene is used and crop plants containing the *kan*^{r} gene will be produced. Specifically, this section addresses the physical containment procedures to prevent escape of: (1) plant vectors containing *kan*^{r} genes (i.e., *Agrobacterium tumefaciens*), (2) source organisms containing *kan*^{r} genes (i.e., *Escherichia coli*), and (3) crop plants containing the *kan*^{r} genes (i.e., tomato, cotton, or rapeseed), from the laboratory, greenhouse, or field. These containment procedures are designed to reduce the potential for environmental exposure and to reduce the potential for exposure to persons outside the laboratory, greenhouse, or specific field trial site. In addition, these procedures limit exposure to persons working within the laboratory, greenhouse, or field.

Plant material and microorganisms containing the *kan*^{r} and gene product are all treated and disposed of in accordance with containment and disposal procedures discussed here and provided in Appendix EA-7.

The only potential emission that may occur is pollen from greenhouses. However, the principle means of pollen movement with cotton, rapeseed and tomato is via insects. Greenhouse containment is designed to eliminate insect movement into and out of the greenhouses. Isolation distance appropriate for containment are maintained around greenhouses to prevent cross-pollination with compatible crops and/or wild relatives in the rare event that pollen should escape greenhouse containment. Additionally, plant surveys are routinely conducted around greenhouses to ensure no related plant species are present. If relatives are found, they are destroyed

In 1986, the National Institutes of Health (NIH) established guidelines for laboratories that perform recombinant DNA research. These guidelines entitled, "Guidelines for Research Involving Recombinant DNA Molecules" (FR 51, No. 88, May 7, 1986), were designed to supplement existing biological safety programs already in place at most microbiological laboratories. In these guidelines, NIH presents no estimates of the risk of exposure to or escape of recombinant DNA organisms once the specified containment conditions are implemented.

The following discussion is a brief summary of the NIH guidelines and their implications for this EA. As NIH states, the main objective of a biological safety program is to confine organisms containing recombinant DNA

molecules and thus reduce the potential for exposure of the laboratory worker, persons outside the laboratory, and the environment in general to organisms containing recombinant DNA molecules. Effective biological safety programs rely on three mechanisms: (1) a set of standard practices that are generally used in all microbiological laboratories, (2) special procedures providing physical containment barriers that are applied to varying degrees according to the estimated biohazard of the recombinant DNA organism, and (3) the application of highly specific biological barriers. The ensuing discussion deals specifically with physical containment barriers.

For physical containment, NIH has established four biosafety levels (BL) for work involving recombinant DNA, with BL4 providing the most stringent containment conditions and BL1 providing the least stringent conditions. These biosafety levels consist of combinations of laboratory practices and techniques, safety equipment and laboratory facilities appropriate for the operations performed and the hazard posed by the identified agents. Descriptions and assignments of physical containment are based on existing approaches to containment of pathogenic organisms. The National Cancer Institute (NCI) describes 3 levels for research on oncogenic viruses which roughly correspond to the NIH's BL2, BL3 and BL4.

Calgene currently maintains a BL1 laboratory facility for its work with recombinant DNA. The classification of Calgene's laboratory and greenhouse research involving recombinant DNA as BL1 is based on an evaluation of several factors including:

- Source of DNA, host and vector;
- Non-pathogenicity of the genes and organisms involved;
- Low potential hazard posed to humans, animals, plants and the environment;
- Laboratory practices and techniques; and
- Safety equipment and laboratory facilities available for use.

In addition, Calgene's laboratory and greenhouse facilities have been inspected and determined to be in compliance with BL1 containment by the USDA and the California Department of Food and Agriculture (CDFA). The specific laboratory procedures that Calgene has implemented to comply with BL1 containment are listed in Appendix EA-7.

In 1989, the U.S. Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP) developed guidelines for physical containment procedures in greenhouse facilities where experimental microorganisms are being

developed. These guidelines describe four levels of physical containment, GH1 through GH4. Level GH4 provides the most stringent containment conditions while GH1 provides the least stringent conditions. Many of the methods employed for physical containment of microorganisms in laboratories are believed to be equally valid for greenhouse facilities. Therefore, the NIH laboratory guidelines were used as a model for the development of the EPA greenhouse containment guidelines. In fact, the four levels of physical containment described for greenhouses are essentially analogous to the four physical containment levels outlined by NIH for laboratories. On June 16, 1987, the USDA APHIS published their policy on "Plant Pests; Introduction of Genetically Engineered Organisms or Products; Final Rule" in the Federal Register (52:22892-22915). Calgene also uses these guidelines for greenhouse containment and periodically provides APHIS with an updated BL1 greenhouse containment guideline. Calgene currently maintains a GH1 greenhouse facility for its work with plants containing recombinant DNA. The specific greenhouse containment procedures that Calgene has implemented to comply with the NIH, USDA and EPA guidelines are listed in Appendix EA-7.

For contained field trials of genetically engineered plants, Calgene uses the USDA APHIS "Plant Pests; Introduction of Genetically Engineered Organisms or Products; Final Rule" in the Federal Register (52:22892-22915). This rule requires a permit from USDA APHIS to import, move interstate or release into the environment under contained conditions, a genetically engineered organism or product. The rule provides specifications for container requirements during movement and storage and requires permit applicants to provide "a detailed description of the proposed procedures, processes and safeguards which will be used to prevent escape and dissemination of the regulated article." To date, Calgene has received 52 field trial and over 100 movement permits from the USDA APHIS. In order to maintain appropriate containment, Calgene has produced "Field Trial Notebooks" for cotton, rapeseed and tomato (Appendix EA-8). These notebooks are used by Calgene staff and cooperators to ensure compliance with Federal and State regulations for field trials and to prevent uncontained release of genetically engineered plants into the environment. Contained within the notebooks are the specific safeguards Calgene employed to contain the transgenic plants within the field trial site. The notebooks are also used to record observational data on transgenic plants in the field to determine if the plants have any abnormalities, crown gall (*A. tumefaciens*) disease symptoms, or unusual germination, flowering, or other developmental characteristics.

On March 31, 1993, the USDA APHIS published a new rule to amend the previous rule on introduction of genetically engineered organisms: "Genetically Engineered Organisms and Products; Notification Procedures for the Introduction of Certain Regulated Articles; and Petition for Nonregulated Status; Final Rule" in the Federal Register (58:17044-17059). This amendment

allows the use of a notification procedure for movement and contained field trials of genetically engineered plants using performance standards to prevent release into the environment. The amended rule at this time is limited to six crops: corn, cotton, potato, soybean, tobacco and tomato. Calgene is now using the notification procedures for its transgenic cotton and tomato.

F.3 State and Local Regulations

Calgene is in compliance with a wide variety of State and local regulations that are applicable to Calgene's laboratory, greenhouse and field operations. Additionally, Calgene holds several permits for the disposal of materials used in laboratories during the development of *kan*^{r} containing organisms. Those regulations and permits listed below pertain to some aspect of the production of transgenic tomatoes, cotton, or rapeseed containing *kan*^{r} genes. Operations range from laboratory analyses involving chemicals or radioisotopes to plant growth activities in greenhouses.

Calgene is in compliance with the following agencies and regulations:

California Department of Food and Agriculture

California Department of Industrial Relations

- Title 8 (Occupational Health and Safety): Comply with general duty clause to provide a safe and healthful work environment as well as specific requirements relating to laboratory and greenhouse operations (i.e., use of engineering controls, personal protective equipment and administrative controls).

State of California Water Resources Control Board

- Title 23: Comply with all discharge requirements and restrictions.

State of California Air Resources Board

- Title 17 (Sections 90700 through 94145): Comply with all requirements associated with emissions and pollution control.

California Department of Health Services

- Title 22 (Hazardous Waste Management):

  Biological Waste (Sections 66835 through 66850) - Comply with all requirements for the generation, storage, treatment and disposal of biological wastes (e.g., bacterial or transgenic plant materials).

  Chemical Waste (Sections 66330 through 66944) - Comply with all requirements for the generation, classification, handling and disposal of hazardous chemical waste.

- Title 17 (Radiation Control): Comply with all requirements of our State License for Radioactive Materials Use including personnel and area monitoring, inventory control, disposal and record keeping.

- Proposition 65 (California's Safe Drinking Water and Toxic Enforcement Act of 1986): Comply with all notification and posting requirements for materials covered by this law.

North Carolina Department of Agriculture

- Genetically Engineered Organisms Act, Chapter 106, Article 64 of the General Statutes of North Carolina.

- Regulations for Genetically Engineered Organisms, Title 2, Chapter 48, Subchapter 48E of the North Carolina Administrative Code.

Local Regulations and Ordinances

- Comply with all local regulations and ordinances including Hazardous Materials Inventory and Business Plan submission to the City of Davis Fire Department as part of "Community Right-to-Know," City of Davis Waste Water Discharge Ordinance and County Agricultural Commission requirements for disposal of pesticide containers.

Calgene has been issued and is in compliance with the following permits:

- State of California, Department of Health Services, Radioactive Materials License #3849-57.

  Applies to specific isotope usage and a decay program for ^{32}P.

- City of Davis Pre-Treatment Permit Application Form: July 1990 Letter.

  Discharge of water from cooling towers and assurance that no chemicals enter waste water.

- California Environmental Protection Agency Department of Toxic Substances Control, Extremely Hazardous Waste Permit #1-4074.

  Notification to California if any waste in this category disposed of.

- United States Environmental Protection Agency Generator #CAD047140280.

  Small quantity generator for all hazardous waste. All waste is non-RCRA.

F.4 Introduction of Substances into the Environment: Production of Tomato Seed, Cotton Seed and Rapeseed Seed.

This section of the EA discusses the production of tomato, cotton and rapeseed during scale-up for seed increase and commercial use, addressing applicable items in 21 CFR 25.31a(a).

Sites of Production. Field production locations are typical growing regions for cotton, rapeseed and tomato (see D.4).

List of substances expected to be emitted. Only APH(3')II is produced by the *kan*^{r} gene. The *kan*^{r} gene is contained in the cotton, rapeseed and tomato chromosomes in each living cell in the plant. The gene product, aminoglycoside 3'-phosphotransferase II (APH(3')II), is produced constitutively and is found throughout the plants. Products from the crops (seeds, cotton and rapeseed meal and tomato fruit) and plant debris may contain the *kan*^{r} gene and gene product. Rapeseed and cottonseed oil do not contain detectable levels of either the *kan*^{r} gene or the gene product APH(3')II.

Controls exercised. No additional controls are warranted during the commercial production of tomato, cotton and rapeseed containing the *kan*^{r} gene relative to the production of such crops that do not contain the *kan*^{r} gene. Tomato, cotton and rapeseed plants will be grown using agricultural practices appropriate for the regions of the country where the crops are commercially produced (see D.4). This includes standard practices for seed production addressed in the Association of Official Seed Certifying Agencies (AOSCA) Handbook (1971). Appropriate agricultural practices will be used for harvesting and removal of crop plants and to prepare fields for subsequent crop production.

Citation of and statement of compliance. Calgene is currently in compliance with all applicable emissions requirements (include occupational) at the Federal, State and local level. Any use of pesticides will follow EPA label registration. Approval of this Food Additive Petition will not change this compliance status.

Compliance with current emissions requirements. Currently, there are no emission requirements for the *kan*^{r} gene and APH(3')II. The approval of this proposed action will have no effect upon compliance with current emissions requirements at the production sites.

Quantities and concentrations of substances. Estimates of the quantities and concentrations of the *kan*^{r} gene and APH(3')II expected to enter the environment as a result of use and disposal of products are presented in sections G.2.1 and G.2.2.

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Section G. Fate of Emitted Substances in the Environment

Emission of APH(3')II into the environment is of no concern. APH(3')II is not a toxin or allergen. Enzymes, which include APH(3')II, generally do not raise safety concerns since very few toxic agents have enzymatic properties (Pariza and Foster 1983). It is very unlikely that the *kan*^{r} gene which encodes APH(3')II could move from the plant genome via horizontal gene transfer; certainly, no mechanism of such transfer has been demonstrated. Even if such transfer could occur, there would still be no concern of emission of APH(3')II into the environment, since calculations (Appendix EA-9) using the most favorable assumptions and probabilities demonstrate lack of significant changes in the potential numbers of microorganisms containing the *kan*^{r} gene or potential impact on the level of APH(3')II in the environment. This section of the EA presents data to support these conclusions.

Once the crop plants containing the *kan*^{r} genes have been cultured in the laboratory and cultivated in the greenhouse, extensive field testing is performed to select individual transgenic events that perform well agronomically and in terms of product quality. Progeny of selected events are grown for further testing and to increase seed. Finally, these transgenic crop plants will be planted and cultivated in commercial fields. This section of the EA evaluates the potential risk of moving crop plants containing *kan*^{r} genes from a contained research environment (i.e., laboratory, greenhouse, or small-scale field trial) to an uncontained commercial environment. The two potential concerns arising from the commercial growing of crop plants containing antibiotic resistance genes are: (1) the fate of the *kan*^{r} gene and (2) the fate of APH(3')II.

Relative to the environmental impact of released genetically engineered microorganisms, Stotzky (1989) states, "Preliminary studies on the effects of adding to soil high concentrations (e.g., 10^8 CFUs per gram of soil) of various strains of *E. coli*, *E. cloacae* and *P. aeruginosa*, with and without plasmids carrying antibiotic-resistance genes, have not shown any consistent and lasting effects on the gross metabolic activity (as measured by CO_2 evolution), transformation of fixed nitrogen, activity of soil enzymes (phophatases, arylsulfatases, dehydrogenases) and species diversity of the soil microbiota." This statement lends support to the conclusion that the introduction of crop plants containing an antibiotic-resistant selectable marker is not likely to result in adverse environmental impacts.

In their review of selectable marker genes, Flavell et al. (1992) concluded the following:

- The NPTII (*kan*^{r}) gene product (APH(3')II) is not toxic to humans or animals.
- Eating APH(3')II does not compromise oral kanamycin and neomycin therapy in humans.

- Transfer of the gene from plant to pathogenic bacteria to humans or other species is highly unlikely and would not compromise antibiotic therapy in humans or animals if it did happen.
- Potential gene movement from genetically modified plants will not cause unacceptable environmental damage.
- "Overall, the ubiquity of the gene in nature and its benign properties make it ideal as a selectable marker in plant transformation."

In their review on the biosafety of kanamycin-resistant transgenic plants, Nap et al. (1992) concluded:

- "The [*kan*^{r}] gene used for resistance is an excellent choice because of the high substrate specificity of the enzyme encoded."
- "Human or veterinary antibiotic therapies will not be compromised. ... At no point will there be any interference with modern antibiotic therapies. Even the probability that the *eu-apha2* [*kan*^{r}] gene would interfere with the efficacy of the veterinary applications of kanamycin and neomycin is negligible."
- "The physico-chemical characteristics of the antibiotic exclude the existence of selective conditions in the environment. Therefore, a transgenic plant or any other organism that might have acquired the gene will not get any selective advantage because of this gene."
- "There is no toxicity or predictable harm of both gene or gene product for human or animal consumption."
- "Full legislative clearance of this transgenic trait is therefore acceptable."
- "Kanamycin-resistant transgenic plants can generally be recognized as safe."

In his review on the ecology of transgenic plants, Crawley (1992) concluded:

- "There is a view that the ecology of genetically engineered organisms is somehow different from the ecology of conventional organisms, and that the intentional release of genetically engineered organisms poses a greater threat to the Balance of Nature than do other kinds of organisms bred by man. This view is mistaken. ... The ecological rules are the same for transgenics as for non-transgenics."
- "Release of genetically engineered crop plants does not pose substantial new threats to the environment.

Antibiotic resistance genes. One consideration for the fate of antibiotic resistance genes is that they might be transferred to microorganisms and confer antibiotic resistance to these organisms. The model presented in Appendix EA-9 suggests that transfer of the *kan*^{r} gene from transgenic plant materials to soil bacteria would only happen very infrequently, if at all; even though the model is built on assumptions including efficient uptake and expression in strains of the receptive bacterial genus *Bacillus*. In addition, the absence of direct selection for the APH(3')II gene product and the current background of various kinds of kanamycin-resistant bacteria in combination with the low abundance of those

resistant bacteria in relation to non-resistant species in soils indicate that newly resistant strains would not gain a competitive advantage over existing strains. We conclude, therefore, that even if the *kan*^{r} gene were transferred to microorganisms in the soil, this would have no significant environmental impact.

It has been reported that strong selective pressures for developing and maintaining antibiotic resistance in the natural environment do not exist (Stotzky and Babich 1986, Stotzky 1989). For example, it has been shown that Tn*5*-containing *Rhizobium* has no competitive advantage over its wild-type parent in soil (Pillai and Pepper 1990) and kanamycin-resistant *Erwinia* has no competitive advantage over its wild-type parent in water (Scanferlato 1989). These experiments suggest that there is not a significant selective pressure in these environments to confer a competitive advantage to Tn*5*-containing organisms over their "wildtype" counterparts.

Kanamycin-resistant microorganisms occur naturally in the environment (Henschke and Schmidt 1989, Van Elsas and Pereira 1986), these kanamycin-resistant soil microorganisms constitutively produce gene product (Davies 1986), and at least some of this gene product appears to be APH(3')II (Leff et al. 1993). Resistance due to APH(3')II production is only one of various mechanisms which can render soil microorganisms resistant to kanamycin.

A first mechanism is that many bacteria exhibit natural resistance to antibiotic substances simply because they fail to take up the antibiotic (reviewed in Benveniste and Davies 1973, Foster 1983). The failure to take up an aminoglycoside can be an inherent feature of the cell wall unrelated to kanamycin per se. Thus, even in the absence of antibiotic selection, naturally occurring kanamycin-resistant bacteria are common in soil samples.

A second mechanism of naturally occurring aminoglycoside resistance in soil bacteria is evident in the methylation of the 50S rRNA, which has been observed in a wide range of soil bacteria (Tanaka and Weisblum 1975). Methylation of this structural RNA molecule confers resistance to a wide spectrum of antibiotics that includes kanamycin (Foster 1983). Thus, naturally occurring kanamycin-resistant bacteria may result from selection due to another antibiotic; this represents a direct selection for kanamycin-resistance in which the APH(3')II offers no adaptive value.

A third mechanism for kanamycin resistance is evident in the soil actinomycetes. Bacteria that naturally produce aminoglycosides related to kanamycin have been found to contain aminoglycoside-inactivating enzymes as internal protective mechanisms (reviewed in Benveniste and Davies 1973, Cundliffe 1989, Foster 1983, Shaw 1993). Thus, such bacteria naturally occur in soil samples, contain genes encoding aminoglycoside modification enzymes, and will thrive under kanamycin selection applied artificially. Moreover, the genes

encoding such enzymes are widely believed to be the primary source of genes conferring aminoglycoside resistance by direct modification of the antibiotic and represent a persistent and continuing source of antibiotic resistance genes in soils. Notably different than the *kan*^{r} gene in transgenic plant tissue, these actinomycete genes are in a form accommodating mobility in procaryotic organisms.

The presence of kanamycin-secreting bacteria in soils suggests that under certain conditions (Appendix EA-10) there may be kanamycin present in natural environments. However, natural kanamycin selection must be slight or distinctly localized in soils as most soil bacteria are kanamycin-sensitive. There are reports indicating that kanamycin resistance genes can be maintained for certain time periods in bacteria in the environment despite the absence of selection (Henschke and Schmidt 1990, Leff et al. 1993). However, since bacteria carrying aminoglycoside modification genes do not accumulate to proportionally high levels in soils, it follows that there is may also be selection for bacteria that does not have these genes (i.e., there normally exists some natural selection against the presence of such genes).

In summary, if environmental conditions in some specific situation did favor increased populations of kanamycin-resistant bacteria, it's clear there are already reservoirs of many different bacterial species - and antibiotic resistance genes - which could respond to those conditions. That kanamycin-resistant bacteria make up such a minute proportion of the total of soil bacteria suggests that the resistance trait does not confer a competitive advantage, as shown by Pillai and Pepper (1990) and Scanferlato (1989). These data, when combined with the quantitative model-derived estimate that gene transfer from plant material to soil bacteria approximates zero (i.e. 10^{-7}-fold increase, see Appendix EA-9), leads to the conclusion that transgenic crop plants containing the kanamycin resistance gene will have no impact on antibiotic resistance patterns in soil microorganisms.

In its "Interpretive Ruling on Calgene, Inc., Petition for Determination of Regulatory Status of FLAVR SAVR™ Tomato," the USDA APHIS concluded that it "has not identified any potential effects arising from any rare gene transfer from FLAVR SAVR™ tomatoes to microorganisms, should any occur" (Appendix EA-11).

Antibiotic resistance gene products. The concern with the fate of antibiotic resistance gene products is that as a consequence of the increase in antibiotic resistance genes, gene product (i.e., APH(3')II) could reach concentrations in the environment that may be toxic to plants, soil microflora, or wildlife. However, this is not likely due to the lack of persistence and low toxicity of most proteins (i.e., gene products) in the environment and due to the narrow substrate specificity of most enzymes, including APH(3')II (Nap et al. 1992). In general, natural organic materials are rapidly degraded in the soil, usually within three

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weeks (Tisdale and Nelson 1975). Furthermore, enzymes like APH(3')II generally do not raise safety concerns since very few toxic agents have enzymatic properties (Pariza and Foster 1983). Also, APH(3')II is not and has never been intended to be used as a toxic agent in the same manner that pesticides or herbicides are used.

In addition, since kanamycin-resistant microorganisms occur naturally in the environment (Henschke and Schmidt 1989, Van Elsas and Pereira 1986; see above), these kanamycin-resistant soil microorganisms may constitutively produce gene products (Davies 1986), and at least some of this gene product is likely APH(3')II (Leff et al. 1993). Therefore, organisms are most likely being exposed to a certain, constant low-level background of APH(3')II or related enzymes. These background levels are relatively low because of the low prevalence of kanamycin-resistant microorganisms in the soil (Van Elsas and Pereira 1986). However, no toxicity has been associated with this low-level background exposure. Nap et al. (1992) concluded that, "there is no toxicity or predictable harm of both [*kan*^{r}] gene or gene product [APH(3')II] for human or animal consumption."

Agricultural worker exposure. A final concern of environmental release is exposure of agricultural workers to crop plants containing the *kan*^{r} gene as these crops are being grown commercially in the field.

We have concluded that exposure of agricultural workers to the *kan*^{r} gene and APH(3')II via dermal contact with plants or inhalation of plant material poses no additional health risk to these workers and will be insignificant compared to exposure via ingestion. This conclusion is based on our understanding of the structure, biochemical nature and mode of action of the APH(3')II enzyme.

Exposure in this subpopulation would be limited to plant debris (e.g. pollen and dust) containing *kan*^{r} genes and/or gene product (APH(3')II) generated as a result of plant cultivation and harvesting activities. The growing of transgenic tomatoes, cotton and rapeseed containing the *kan*^{r} gene will be identical to the growing of the same non-transgenic crops. The plant debris to which workers will be exposed will therefore be the same in both situations. In addition, workers are already exposed to *kan*^{r} genes found naturally in soil microorganisms.

Human exposure via plant debris is estimated to be insignificant relative to human exposure via ingestion of *kan*^{r} genes and/or gene products through food consumption. Ingestion provides significantly more exposure of the *kan*^{r} gene and APH(3')II than via plant debris. Data on exposure through ingestion were previously submitted in Volume 1 of this petition and are again included here as Appendix EA-12 in support of the following conclusions (see also Appendix EA-13 and Nap et al. 1993):

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1) Ingestion of plant material containing the *kan*^{r} gene will not compromise the efficacy of antibiotics to which the *kan*^{r} gene confers resistance.

2) Ingestion of plant material containing APH(3')II will not compromise the efficacy of antibiotics that could be inactivated by the enzyme.

3) Ingestion of plant material containing APH(3')II will not result in direct toxicity.

4) APH(3')II is degraded under simulated human gastric and intestinal conditions.

5) APH(3')II does not have significant homology with known toxins or allergens.

6) The *kan*^{r} gene has been used safely in human gene therapy.

Small-scale field trials. In evaluating the risk of environmental release, useful empirical information concerning transgenic plants has been obtained from numerous field trials which have demonstrated the safety of the transgenic plants and supported the conclusions made by the USDA APHIS in their Environmental Assessments of the field trials. In this section, the EAs prepared by USDA and the resultant field trial reports and data on transgenic crops tested by Calgene are discussed.

The USDA has issued a total of 150 permits for field trials of transgenic cotton, rapeseed and tomato (Appendix EA-14). All the EAs prepared by the USDA for these trials resulted in findings of no significant impact (FONSI). Forty-six of the 150 permits were issued to Calgene. Prior to initiation of Calgene's small-scale field trials for FLAVR SAVR™ tomato, bromoxynil-resistant cotton and oil-modified rapeseed, the USDA APHIS prepared EAs evaluating available scientific information related to the introduction of genetically engineered plants into the environment. These EAs were in response to permit requests made by Calgene to perform controlled field tests and are required under Title 7 of Federal Regulations, Part 340. The purpose of the EAs is to "describe ways in which risk to the environment is limited either by the nature of the organisms or by specific safeguards that have been designed into the field test protocol." Example EAs for cotton, rapeseed and tomato prepared by USDA are included as Appendix EA-15.

The key points, as related to environmental impact, discussed by USDA in each EA can be summarized as follows:

- "There is no evidence that cross-pollination with other plants in the study plot will occur. Two crops (tomato and cotton) are predominantly self-

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pollinating, the nature of cotton pollen does not favor wind dispersal and there are no sexually compatible plants close to the respective study sites. The third crop (rapeseed) does outcross with other, non-transgenic rapeseed plants and to closely related relatives. However, appropriate techniques, such as the use of border rows or physical isolation and monitoring, were employed in the field test to prevent the occasional or accidental cross-pollination by insects to plants outside the contained field trials."

- "Once the gene is inserted into the plant genome the vector does not survive. Therefore, the gene is stably incorporated and no mechanism is known to move it to any sexually incompatible plant."

- "There is no identified factor of these field tests that will be harmful to vertebrate or invertebrate fauna. There is no evidence that beneficial insects will be affected."

- "There is no evidence that there will be an effect on the plant's susceptibility to disease (i.e., fungal, bacterial or viral)."

- "There is no evidence that the altered genes would give the plant any selective advantages in the environment."

Therefore, the USDA issued a finding of no significant impact (FONSI) for each trial and concluded that there is no reason to believe that 1) genetic alterations will spread to other plants in the field site; 2) native fauna will be affected; or 3) niches established by the plant itself, its beneficial insects, or its diseases will be altered in any way.

Field trial reports have been produced by Calgene for transgenic cotton, rapeseed and tomato (Appendix EA-16) as follows:

- Bromoxynil-tolerant cotton

- Oil-modified rapeseed (high stearate and laurate)

- FLAVR SAVR™ (antisense polygalacturonase) tomato

Observations and data collected from these field trials and laboratory tests confirm the USDA EA conclusions. The containment conditions used in each of these trials was sufficient to prevent cross-pollination outside the trials and there was no evidence in any of the trials that propagules of the transgenic plants were disseminated. None of the transgenic plants expressed any plant pest characteristics. The inserted genes were stably incorporated into the plant genome and maintained in subsequent generations in a Mendelian fashion. The transgenic plants did not affect vertebrate or invertebrate fauna nor beneficial insects. The transgenic plants did not have increased susceptibility to disease.

No selective advantages were observed in any of the transgenic plants (Appendix EA-16, Kiser and Mitchell 1991, Kramer et al. 1990, Kramer et al. 1992).

Tomato. For tomato, the following data were generated which support the conclusion that tomato containing the *kan*^{r} gene and other inserted genes do not differ from nontransgenic tomato in their potential environmental affects (Appendix EA-3):

- Seed germination rate and frequency of FLAVR SAVR tomatoes is equivalent to traditionally bred varieties. FLAVR SAVR tomatoes have been used for breeding purposes; no changes in flowering time, no improved outcrossing characteristics, no changes in seed production and no changes in controlled pollination occurred. Yield of FLAVR SAVR tomatoes is equivalent to traditionally bred controls. Current agricultural practices will not be affected by cultivation of the FLAVR SAVR tomato.

- The FLAVR SAVR tomato differs from other tomato cultivars only in terms of characteristics related to pectin and the presence of the novel *kan*^{r} gene and APH(3')II gene product. Nutritional levels, taste (for tomatoes picked at the same stage), processing characteristics, horticultural and developmental traits and potential toxins (solanine and tomatine) are unchanged, except for those related to pectin.

- FLAVR SAVR tomatoes were shown to be genetically stable. The inserted genes were shown to be immobile and segregated according to Mendelian predictions. Levels of APH(3')II, polygalacturonase, and vitamins A and C were stable in different generations.

Cotton. For cotton, the following data were generated which support the conclusion that cotton containing the *kan*^{r} gene and other inserted genes do not differ from nontransgenic cotton in their potential environmental affects (Appendices EA-14, EA-16 and EA-17):

- The BXN cotton differs from other cotton cultivars only in terms of characteristics related to resistance to the herbicide bromoxynil and the presence of the novel *kan*^{r} gene and APH(3')II gene product. Lint yield, lint percent, fiber strength, lint length, lint elongation, maturity rating, micronaire, and fiber characteristics were within the ranges for control, commercial varieties.

- BXN cotton varieties showed no change in seed dormancy characteristics. BXN cotton varieties have been used for breeding purposes; no changes in flowering time, no improved outcrossing characteristics, no changes in seed production and no changes in controlled pollination were observed.

[redacted]

- BXN cotton varieties were shown to be genetically stable. The inserted genes were shown to be immobile and segregated according to Mendelian predictions.

- No *Agrobacterium* disease symptoms have been observed in cotton in the field.

Rapeseed. For rapeseed, the following data were generated which support the conclusion that rapeseed plants containing the *kan*^{r} gene and other inserted genes do not differ from nontransgenic rapeseed in their potential environmental affects:

- The High Stearate rapeseed did not differ from traditionally bred varieties in percent seed germination, stand establishment, days to flowering, days to maturity or seed yield (Appendix EA-16, Rapeseed Field Trial Reports). The *kan*^{r} gene and oil modification genes do not affect fertility, do not alter visitations by pollinating bees and do not increase outcrossing frequency (Appendix EA-18, Issue 6).

- The High Stearate rapeseed exhibits some characteristics in the seed/seedling phase that should give it a selective disadvantage relative to traditionally bred varieties: seedling vigor is reduced and the seeds are more sensitive to the killing effects of temperature extremes (e.g. 0 °C and 35 °C). Field studies indicate that seed survival in the soil is unchanged, while laboratory studies indicate that some combination of environmental conditions could delay germination or induce dormancy in some of the surviving High Stearate seed exposed to temperature extremes (Appendix EA-18, Issues 1, 2 and 3).

- As intended, the fatty acid composition of the seed is modified: levels of stearate are significantly increased, primarily at the expense of oleic acid. Levels of the naturally occurring toxicants erucic acid and alkenyl glucosinolates remain within specifications for canola (Appendix EA-18, Issue 1).

- High Stearate rapeseed is genetically stable. The inserted *kan*^{r} gene segregates according to Mendelian predictions. Segregation data is consistent with genetic data from Southern blots of isolated DNA (Appendix EA-16, Rapeseed Field Trial Reports).

- Specific field studies designed to evaluate aspects of weediness potential have been completed by the PROSAMO group in the United Kingdom (Crawley 1992, Cherfas 1991, Scheffler et al. 1992). The studies compared rapeseed containing the *kan*^{r} gene, with its parent *B. napus* rapeseed variety, Westar, and concluded the following:

- In an evaluation of persistence potential, buried seed from transgenic rapeseed lines consistently had greater mortality than the nontransgenic lines, and exposed seed were quickly eaten by small predators.

- The invasive potential of transgenic rapeseed was evaluated under field conditions and found to be non-invasive. The plants did not outgrow their competitors in the wild.

- Transgenic rapeseed was unable to become established and produce seed when sown to undisturbed vegetation in any of twelve experimental sites in the UK over a two year period. Further, even in fertile, disturbed soil the transgenic rapeseed had a net reproductive rate less than 1, meaning that it would become extinct without human intervention.

Summary of small-scale field trials. These field trial data demonstrate that plants containing the *kan*^{r} gene can be produced using current agricultural systems and have shown no unexpected environmental effects. Calgene's cumulative experience base now includes over 15 FLAVR SAVR tomato field trials including three production-scale plantings, over 125 acres of cotton trials harvested between 1989-1992 spanning the US cotton growing region and some 15 acres of rapeseed successfully grown and harvested in our target specialty oil production regions. In all three crops, Calgene has moved beyond the use of small plot research equipment and has managed, planted, cultivated, harvested and transported the crops with standard farm equipment. From fresh market tomato production sites, over 100 tons of tomato fruit have been harvested, sorted, packed, shipped and analyzed for quality traits. Oil and meal samples of cottonseed and rapeseed have been produced in pilot-scale crushing facilities. In preparation for the commercial sale of cottonseed and the production of test market quantities of rapeseed oil, Calgene has produced planting seed that meets established certification requirements. The seed has been cleaned, conditioned and transported using equipment standard to the seed industry. For example, over 3,500 lbs of rapeseed were produced on a 2 acre field in 1992. The seed was harvested, transported, cleaned and stored in certified facilities. In early 1993, approximately 8 tons of breeder cottonseed was delinted, prepared for planting, shipped to seed increase sites in four states and planted on more than 300 acres for further seed increase. Based on our experience, the presence of the *kan*^{r} gene and gene product will have no effect on scale-up to commercial production.

Deregulation of genetically engineered plants by USDA APHIS. On March 31, 1993, USDA APHIS published its "Genetically Engineered Organisms and Products; Notification Procedures for the Introduction of Certain Regulated Articles; and Petition for Nonregulated Status; Final Rule" in the Federal Register (58:17044-17059). This rule provides specific procedures for petitioning the USDA to determine that a genetically engineered plant is no longer a plant

pest risk and therefore no longer regulated under 7 CFR 340. Once such a determination is made, then the genetically engineered crop can be grown uncontained in the field just as any other agricultural crop.

Deregulation of Calgene's FLAVR SAVR™ tomato. On October 19, 1992, the USDA APHIS issued an interpretive ruling that the FLAVR SAVR™ tomato "does not present a plant pest risk and is not a regulated article under the regulations contained in 7 CFR part 340. This action is in response to a petition submitted by Calgene, Inc. seeking a determination from APHIS that its FLAVR SAVR™ tomato no longer be deemed a regulated article based on an absence of plant pest risk. The effect of this action is that Calgene's previously field tested lines of the FLAVR SAVR™ tomato and their progeny using one of seven binary vectors and the FLAVR SAVR™ gene with its associated promoter and terminator are no longer subject to regulation under these regulations" (Appendix EA-11).

The USDA APHIS held a 45-day public comment period on their interpretive ruling and received a total of 19 comments, 15 of which were in support and 4 of which had serious reservations about or disapproval of the ruling. In response to the negative comments, APHIS determined that it possessed sufficient information to make its ruling from data generated from eight FLAVR SAVR™ tomato field trials conducted between 1988 and 1992. APHIS agreed that there could be passive movement via pollen of the introduced traits from FLAVR SAVR™ tomatoes into other cultivated tomatoes, but identified no indications of such low level movement that would differ from that for any other tomato gene (Appendix EA-11).

APHIS concluded (Appendix EA-11),

- "Neither the introduced genes, their products, nor the added regulatory sequences controlling their expression presents a plant pest risk in these FLAVR SAVR™ tomatoes."

- "FLAVR SAVR™ tomatoes have no significant potential to become successful weeds."

- "The FLAVR SAVR™ tomato will not increase the weediness potential of any other plant with which it can interbreed."

- "The FLAVR SAVR™ tomato will not cause damage to processed agricultural commodities."

- "The FLAVR SAVR™ tomato will not be harmful to beneficial organisms, including bees."

The result of this interpretive ruling is that Calgene can conduct interstate movement and field trials of FLAVR SAVR™ tomatoes without any restrictions that are not applied to any other tomato cultivars. The basis for this ruling is that none of the introduced genes or their products could result in significant environmental impact once the FLAVR SAVR™ tomatoes were scaled-up and grown in uncontained production fields.

Calgene's BXN cotton. BXN (bromoxynil resistant) cotton was developed by Calgene to improve weed management practices for cotton production. The BXN gene encodes the protein nitrilase which detoxifies the broadleaf herbicide Buctril®, containing the active ingredient bromoxynil. As a result, Buctril herbicide can then be sprayed "over the top" for broadleaf weed control in BXN cotton fields. Calgene intends to petition the USDA APHIS to determine that the BXN cotton is no longer a regulated crop under 7 CFR 340. This petition is based on the following:

- **Cotton is not a weed.** The cotton genus *Gossypium* is not a weed pest risk (Weed Science Society of America 1989). There is very little likelihood that BXN cotton will have enhanced weediness traits compared to non-transformed cotton. Field trials to date demonstrate no propensity for increased weediness (Appendix EA-16). *Gossypium* was not listed as a weed in the following major references: Crockett (1977), Holm et al. (1977), and Muenscher (1980). It is not present on the lists of noxious weed species distributed by the State of California and the Federal Government (1990). In addition, cotton does not contain attributes associated with weed pests (Keeler 1989). The USDA has concluded in environmental assessments of transgenic field trial applications that cotton does not display significant potential to develop into a weed itself and that "the genus shows no particular weedy aggressive tendencies" (Appendix EA-15). Consequently, transformed cotton should have no increased tendency to convert to a weedy state since BXN cotton possesses the same agronomic characteristics as other cotton varieties, which have never been considered weeds, even in agricultural fields.

- **Cotton is not compatible with weed pests.** There is little risk of genetic transfer to other *Gossypium* species because of natural outcrossing barriers and ploidy differences. Upland cotton, *G. hirsutum*, is sexually compatible only with *G. barbadense* and *G. tomentosum* in the United States, neither of which is considered a weed pest (Fryxell 1984, Endruzzi et al. 1984, Fryxell 1979, and National Research Council 1989). Therefore, there is no potential for exchange of weedy traits by cross-pollination with weeds or weedy relatives.

- **Introduced genetic sequences do not alter weediness characteristics.** Genetic sequences from USDA APHIS-regulated articles, introduced into cotton, do not confer characteristics that would present BXN cotton as a plant pest risk (e.g., cause cotton to become a weed pest risk). The BXN and *kan*^{r} genes will

not confer weediness to cotton nor confer any selective advantage which would enhance survival outside an agricultural environment. In its environmental assessments, the USDA concluded, "neither the introduced genes, nor their gene products, confers on cotton any plant pest characteristics" (Appendix EA-15).

- **Dispersal of the *kan*^{r} gene or gene product in environment.** Calculations were made for potential release of the *kan*^{r} gene and gene product, APH(3')II, in the environment (Appendix EA-9). The calculations show that even under the worst case, uptake, incorporation and expression of the gene would not be significant, since the numbers of naturally occurring kanamycin resistant microbes in the soil environment would continue to exceed any newly resistant forms by 7 orders of magnitude. Dispersal of the gene product, APH(3')II is also not a concern, since the enzyme is substrate specific, the enzyme requires ATP for activity, and the antibiotics it acts upon are not used commercially in the environment.

- **The introduced genetic sequences do not alter agronomic characteristics.** Seed germination rate and frequency of BXN cotton were equivalent to traditionally bred varieties. No changes in flowering time, no change in outcrossing characteristics, no changes in seed production, and no changes in controlled pollination were measured. Yield of fiber from BXN cotton was equivalent to traditionally bred controls. No adverse pleiotropic traits (i.e., insertional mutagenesis, the inactivation of host genes into which the incoming DNA is inserted) have been detected to date in BXN cotton selected for agronomic characteristics. Except for weed management, current agricultural practices will not be affected by cultivation of the BXN cotton (Appendix EA-16). Calculations were made to estimate levels of the *kan*^{r} gene product APH(3')II which could be released into the environment under non-contained, scaled-up cotton production. These data are discussed in Section G.2.

- **No new hazardous or deleterious compounds are produced.** No new compounds have been detected in BXN cotton that pose a hazard or are deleterious to the environment. The *kan*^{r} gene isolated from Transposon Tn5 encodes the enzyme aminoglycoside 3'-phosphotransferase II (APH(3')II). This enzyme has a narrow substrate specificity and catalyzes an ATP-dependent phosphorylation of the 3'-hydroxyl group on the aminohexose moiety of several aminoglycoside antibiotics. APH(3')II is produced in kanamycin-resistant microorganisms which occur naturally in the environment (Henschke and Schmidt 1989, Van Elsas and Pereira 1986)

- **Therefore, BXN cotton varieties do not differ from other cotton varieties, except for resistance to the herbicide bromoxynil.**

Calgene's High Stearate Canola. High Stearate canola was developed by Calgene to allow margarine production using vegetable oil that does not require hydrogenation to be solid at room temperature. This was achieved by reducing production of the canola enzyme, 18:0 ACP desaturase, that desaturates the fatty acid stearate into oleate, a monounsaturated fatty acid. Levels of the enzyme were reduced using antisense technology. Calgene intends to petition the USDA APHIS to determine that High Stearate canola is not a regulated article under 7 CFR 340. The petition is based on the following:

- ***Brassica napus* canola is not a weed or a pest.** *B. napus* is not listed as a weed by the Weed Science Society of America (1989) or in the 1992 Weed Control Manual. *Brassica napus* is the only naturalized *Brassica* that is not noted as a pestiferous weed by Rollins (1981). *B. napus* is not listed in *Weeds of the United States* (Lorenzi and Jeffrey 1987). No *Brassica* is noxious (Federal Noxious Weed Regulation, 7 CFR 360) and none of the *Brassica* species are listed in *The World's Worst Weeds* (Holm 1977).

- **Introduced genetic sequences do not confer any selective advantage that could produce pest or weediness characteristics in canola.** Expert opinion is uniform in assessment of the consequences of modifying seed oils in *B. napus*: genes regulating oil component profiles are not expected to confer a competitive advantage to the transgenic plants and are thought to pose little or no potential risk (Agriculture Canada 1990; USDA 1990). Transfer of oil modification genes to weedy relatives is also not considered to pose a risk, since the genes would not confer any selective advantage to the recipient weeds. Replicated field trials were conducted in California, Georgia and Michigan with the High Stearate canola lines. Traits such as seed germination, seed yield, seedling vigor, etc. were measured in the parental line, other cultivars and numerous transgenic lines. The agronomic performance parameters measured for the transgenic lines were not outside the range of expected values based on the history of the crop (Appendix EA-18, Issue 1). In field studies with buried seed in Georgia and California, the oil modified rapeseed was not more persistent than the parent and loss of seed from the seed bank was quite rapid (half-life less than 2 months)(Appendix EA-18, Issue 3). The High Stearate canola is less likely than the parent canola variety to be able to be weedy or invasive due to slower seed germination, lower seedling vigor and greater sensitivity of seeds to killing effects at temperature extremes (Appendix EA-18, Issue 2).

- **The introduced genetic sequences do not increase outcrossing frequency or compatibility with wild relatives.** Under field conditions, *B. napus* can potentially form hybrids with *B. rapa* (syn. *B. campestris*) and *B. juncea*, including both weedy or naturalized and commercially produced forms. Field experiments demonstrated that the introduced genetic construct does not alter visitations by pollinating bees or increase outcrossing frequency in *B. napus* (Appendix EA-18, Issue 6). Thus, if compatible species are near

enough, some outcrossing will occur, but only at a very low frequency. Crop/weed hybrids are not expected to persist in the field because of poor fertility (even when backcrossed to the weedy parent), lack of any selective advantage of the introduced genes, high seedling mortality and poor seed germination (Appendix EA-18, Issues 5, 7 and 8).

- **Dispersal of the *kan*^{r} gene or gene product in the environment.** Calculations were made for potential release of the *kan*^{r} gene and gene product, APH(3')II, in the environment (Appendix EA-9). Calculations show that the even under worst case, uptake, incorporation and expression of the gene would not be significant, since the numbers of naturally occurring kanamycin resistant microbes in the soil environment would continue to exceed any newly resistant forms by 7 orders of magnitude. Dispersal of the gene product, APH(3')II is also not a concern, since the enzyme is substrate specific, the enzyme requires ATP for activity, and the antibiotics it acts upon are not used commercially in the environment.

- **No new hazardous or deleterious compounds are produced.** No new compounds have been detected in High Stearate canola that pose a hazard or are deleterious to the environment. Levels of the naturally occurring toxicants, erucic acid and glucosinolates, are within required specifications in commercially suitable lines of High Stearate canola. Stearate is a safe food substance with no known toxicity to humans or animals. The stearoyl-ACP desaturase gene in the antisense orientation produces transient RNA. Neither the DNA nor RNA produced are toxic or otherwise harmful (IFBC 1990). The *kan*^{r} gene isolated from Transposon Tn*5* encodes the enzyme APH(3')II. This enzyme has a narrow substrate specificity and catalyzes an ATP-dependent phosphorylation of the 3'-hydroxyl group on the aminohexose moiety of several aminoglycoside antibiotics. APH(3')II is produced in kanamycin-resistant microorganisms which occur naturally in the environment (Henschke and Schmidt 1989; Van Elsas and Pereira 1986).

- **Therefore, High Stearate rapeseed varieties do not differ from other rapeseed varieties in any way that would cause environmental harm.**

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Projected market volume. The projected maximum yearly market volume, in acres of transgenic crop planted, for tomato, cotton and rapeseed are given in Table G-1.

Table G-1. Projected Market Volume for Tomato, Cotton and Rapeseed.

Crop	Total Acres Harvested	5% Market Share as Transgenic	20% Market Share as Transgenic
Tomato	140,000[1]	7,000	28,000
Cotton	12,500,000[2]	625,000	2,500,000
Rapeseed	5,000,000[3]	250,000	1,000,000

[1]USDA (1992) for 1988 harvest in the U.S. (fresh market tomatoes only). These figures vary from year-to year.
[2]USDA (1992) multi-year average in the U.S. .
[3]Mielke (1992). Rapeseed is a new crop in the U.S. with 70,000 acres grown in the 1991/1992 season. However, over 7 million acres were grown in Canada and over 43 million worldwide. For these projections, Calgene used an estimate of 5 million acres of rapeseed production in the U.S.

These figures are marketing estimates and should be evaluated considering the uncertainties associated with these types of projections.

In Section G.1.2. and Section G.2.1., estimates of the total yearly release of the *kan*^{r} gene and APH(3')II, respectively, are calculated for the three transgenic crops of interest. However, instead of using the projected acreage listed above for these calculations, we chose to use the most recently available USDA crop acreage figures for tomato and cotton. For tomato, this figure is 416,000 acres per year and includes both fresh market and processing tomatoes (1988 statistics, USDA 1992). For cotton, the acreage figure is 12.5 million acres per year (multi-year average, USDA 1992). For rapeseed, the projected acreage of 1.0 million acres per year listed above was used. The current USDA-listed acreage for rape was not used because rapeseed is a new crop in the U.S. and its acreage is expected to increase over the next few years as this crop gains acceptance by U.S. farmers and the U.S. market for rapeseed products grows. Therefore, the projected acreage for rapeseed is thought to more accurately reflect future production. The use of these acreage figures results in a substantial overestimate of total yearly release of the *kan*^{r} gene and APH(3')II, but they were selected to develop worst-case assumptions.

G.1 Fate of the *kan*^{r} Gene

This section of the EA discusses the first of the two potential concerns for an environmental release of crops containing the *kan*^{r} gene, transfer and persistence of *kan*^{r} genes into other organisms. The second potential concern is the fate, transport and toxicity of the *kan*^{r} gene product, APH(3')II, which will be discussed in the next section of the EA. Specifically, this section

evaluates the probability of transferring *kan*^{r} genes from crop plants containing the genes to other organisms and the likelihood that the *kan*^{r} gene would provide a selective advantage that would allow it to persist. The four possible routes potentially available for the transfer of *kan*^{r} genes are plant-to-plant, plant-to-bacteria, bacteria-to-bacteria and bacteria-to-plant. Each of these routes will be discussed in turn.

G.1.1 Plant-to-Plant Transfer: Cross-Pollination

Overview. This section of the EA evaluates the transfer of *kan*^{r} genes via cross-pollination of crop plants containing the genes with: (1) neighboring plants of the same species that do not contain the genes or, (2) neighboring "wild" relatives that do not contain the genes.

Cross-pollination is the only known mechanism available for the plant-to-plant transfer of *kan*^{r} genes. Pollen can serve as a vehicle for the movement of *kan*^{r} genes into other plants that are related to the species being cultivated, if they are sexually compatible (Ellstrand 1988). However, significant gene flow by cross-pollination across large distances appears to be fairly limited. Although Ellstrand (1988) presents data to the contrary from studies in wild radishes, it is still generally believed that highly self-pollinating crop species exhibit negligible gene flow at distances beyond 100 meters, while obligate outcrossing crop species do not exhibit appreciable gene flow beyond 1000 meters (Kernick 1961 *as cited in* Ellstrand 1988).

The extent of gene transfer by pollen depends on several factors which include the following (Boyce Thompson Institute for Plant Research 1987):

- Whether the species is naturally self- or cross-pollinated, or both;
- Whether pollen is transmitted by wind, insects, or both;
- Isolation distances from other cultivars of the same species; and
- Proximity of indigenous "wild" relatives and their cross-compatibility.

An additional factor that would limit the spread of *kan*^{r} genes through cross-pollination is the practice of isolating seed production from crop production areas (AOSCA 1971). A nontransgenic crop in production that receives pollen containing the *kan*^{r} gene could produce seed containing the *kan*^{r} gene; however such seed would not be planted the following year, so potential hybrids would not have an opportunity to reproduce and persist. Therefore, accidental hybrids containing the genes would not be propagated to any extent during subsequent growing seasons.

The potential for cross-pollinations is evaluated for tomato, cotton and rapeseed in the latter part of this section. We also discuss the weediness potential in each crop since a primary factor in assessing the environmental impact of a transgenic plant is its "weediness" potential.

The four possible scenarios related to "weediness," three of which are consequences of cross-pollination, are: (1) the crop containing the *kan*^{r} gene could become persistent or invasive (i.e., "weedy"), (2) the crop could transfer its *kan*^{r} gene to neighboring crops through cross-pollination and these crop plants could become weeds, (3) the crop could transfer its *kan*^{r} gene to a "wild" relative through cross-pollination and the resultant hybrid could become "weedy" in natural or cultivated habitats, and (4) the crop could transfer its *kan*^{r} gene to a weed which is by definition "weedy."

In considering "weediness," crops modified by molecular and cellular methods should pose risks no different from those modified by classical genetic methods. Calgene's field trial results demonstrate that cotton, rapeseed and tomato modified using genetic engineering did not increase weediness potential and pose no risks greater than for varieties developed using classical breeding techniques (Appendix EA-16). Molecular genetic methods differ from classical plant breeding methods in that molecular genetic methods involve manipulation of not more than a few genes and associated regulatory sequences. In contrast, classical genetic procedures such as sexual hybridization involve the entire genomes of the parental cells. As the molecular methods are specific in terms of what genes are added, users of these methods are generally more certain about the traits introduced into plants. Preliminary data from conducted field trials confirm the predictable behavior of plants modified by molecular methods and tested in the laboratory and greenhouse (NRC 1989).

In addition, existing procedures for plant breeding, field evaluation and crop certification have evolved to deal with the consequences of genetic recombination leading to undesirable phenotypes. Off-types are routinely removed or rogued as a standard practice. Similarly, off-type transgenic plants would not be carried forward for field introduction, as the goal is to produce an agronomically useful crop plant. As documented in field trial reports submitted by Calgene to the USDA for transgenic tomato, rapeseed and cotton trials (Appendix EA-16), there was no evidence of transformed plants expressing weedy phenotypes or plant pest characteristics. All plants exhibited the normal morphological and growth characteristics expected for the variety which was transformed and regenerated.

The four "weediness" scenarios outlined above assume that the *kan*^{r} gene will confer some type of selective advantage for the plants expressing it.

However, the introduction of an antibiotic resistance trait should confer no selective advantage on a plant in an environment where antibiotics exert no selective pressure. Since seeds of nontransgenic plants are uniformly sensitive to kanamycin, yet are able to germinate and grow in agricultural soils, it is extremely unlikely that levels of kanamycin in soils are sufficient to exert any selective pressure on plants. Evidence suggests that in the absence of selection for antibiotic resistance, the "extra baggage" associated with producing a protein conferring antibiotic resistance actually decreases fitness in the natural environment (Stotzky and Babich 1986). In addition, Stotzky (1989) cites the general lack of "free" antibiotics existing naturally in the soil as evidence for the absence of any strong selective pressure for the development and maintenance of antibiotic resistance. For further discussion of the role of antibiotics in the natural environment, see Appendix EA-10.

Baker (1965 *as cited in* Keeler 1985) noted that there are 12 plant characteristics typically found in "successful" weedy species:

- Germination requirements fulfilled in many environments.
- Discontinuous germination (internally controlled) and great longevity of seed.
- Rapid growth through vegetative phase to flowering.
- Continuous seed production for as long as growing conditions permit.
- Self-compatible, but not completely autogamous or apomictic.
- When cross-pollinated, unspecialized visitors or wind is utilized.
- Very high seed output in favorable environmental conditions.
- Produces some seed in a wide range of environmental conditions; tolerant and plastic.
- Has adaptations for short- and long-distance dispersal.
- If a perennial, has vigorous vegetative reproduction or regeneration from fragments.
- If a perennial, has brittleness, so not easily drawn from ground.
- Has ability to compete interspecifically by special means (rosette, choking growth, allelochemics).

The likelihood that crop plants containing antibiotic resistance genes could independently evolve enough of these traits to become a "successful" weed is extremely low. Although Keeler (1985) proposes that, in certain cases, there may only be a few genes that separate crop plants from weeds, she also concludes that there are no data to suggest that genetically engineered crops are any more likely to evolve into weeds than crops produced by conventional plant breeding methods.

Most crop plants are bred to express a set of traits that result in plants that are agriculturally useful. However, in general, these same plants are not very competitive in the natural environment without significant human intervention (e.g. cultivation and application of fertilizers and herbicides). Therefore, it is doubtful that the addition of a single trait such as antibiotic resistance would result in a crop plant capable of successfully surviving in the natural environment (Boyce Thompson Institute for Plant Research 1987).

G.1.1.1 Tomatoes

Cross-pollination of tomatoes with same species. Tomatoes are considered to be strictly self-pollinating in major U.S. production areas. The domestication (plant breeding) of tomato has selected for enhanced seed set and the propensity for self-pollination. The structure of the flower, the large amount of pollen produced and the limited time of stigma receptivity to pollen all favor self-pollination. The stigma is contained within the floral tube and virtually prohibits outcrossing (Rick 1978). The large amount of self pollen delivered directly to the stigma inhibits cross-pollination. A successful fertilization by foreign pollen results only from the transfer of a large amount of pollen directly to the surface of a receptive stigma. Natural cross-pollination in tomatoes by insect vectors, primarily bees, in the North Temperate Zone has been reported at rates varying between 3 and 30 percent (Rick 1949, Rick *et al.* 1978). Natural cross-pollination rates in Peru, where tomatoes are indigenous, have been reported to be in the neighborhood of 40 to 50 percent (Rick 1950). Both the species of bee and the geographic location of the crop impact the degree of natural outcrossing.

Further evidence for the amount of cross-pollination that occurs in tomato comes from isolation distances established for certification of hybrid plant materials by the Association of Official Seed Certifying Agencies (AOSCA) Handbook (1971). For tomatoes, the required isolation distance from any contaminating source is 200 feet for foundation seed and 30 feet for certified seed.

Calgene has used control pollination techniques to cross transgenic tomato lines containing the *kan*^{r} gene with non-transgenic *L. esculentum* tomato lines to produce hybrids that contain the *kan*^{r} gene. Such breeding practices are common for tomato cultivar development and it is expected that specific genes, such as the *kan*^{r} gene, could be transferred to other tomato lines. In its "Interpretive Ruling on Calgene, Inc., Petition for Determination of Regulatory Status of FLAVR SAVR™ Tomato," the USDA APHIS agreed that there could be passive movement via pollen of the introduced traits from transgenic tomatoes into other cultivated tomatoes, but they identified no implications that such low level movement would differ from that for any other tomato gene (Appendix EA-11).

Calgene has conducted field trials with tomato lines containing the *kan*^{r} gene, but has never observed transfer of the gene to any adjacent, non-transgenic tomato lines (Appendices EA-11 and EA-16).

Cross-pollination of tomatoes with wild relatives. *Lycopersicon* species (i.e., tomatoes) are native to Peru and Ecuador with no wild species occurring in the U.S. In the U.S., *Solanum* species are the most closely related plants to tomatoes and F_1 hybrids have been obtained between *L. esculentum* and *S. lycopersicoides*. However, these hybrids are sterile and no other member of this *Lycopersicon*-like complex has yielded to sexual hybridization with tomato (Stevens and Rick 1986).

Finally, the lack of outcrossing in tomatoes has been further substantiated through personal communications with several experts in the breeding and cultivation of tomatoes (Appendix EA-19). Dr. Charles Rick (tomato geneticist, University of California, Davis) states:

- No probability whatever of tomatoes outcrossing with any native or introduced plants in the U.S. The only intercrossable wild spp. are limited to Latin America.

- Little or no research has been done on minimum pollination buffer distances; furthermore, data obtained for one region might not be applicable to others, since the pollinating bee spp. differ from one region to another. No evidence whatever for wind transport of pollen under field conditions; the responsible agents there are native bees: bumble bees and various groups of solitary bee spp. We have some evidence for wind transport of pollen in greenhouse conditions, but the level is extremely low – something like 1 seed produced from such outcrossing per 50-100 flowers.

Dr. Raymond Clark (USDA, Iowa State University) states:

- I have no reason to suspect you would experience any "escaped" pollen from the California-type tomato material you are working with. We regularly increase our stocks of *L. esculentum* in the field in Ames without protecting our flowers against incoming or outgoing pollen as they are considered to be completely self-pollinating in nature.

- Additionally, there are no native species of plants in the midwest with which tomato will cross, even if tomato pollen were artificially transferred to them. We have over 3,000 accessions of tomatoes in our collection here and have never seen a single outcross to wild species.

Finally, Dr. Jay Scott (plant breeder, University of Florida) states:

- I doubt that tomatoes would cross with any weed species and am aware of no such report.

- In the seed industry, no buffers are used between varieties being increased for seed production. Thus cross-pollination is negligible where stigmas are not exserted naturally or by emasculation.

Therefore, outcrossing of cultivated varieties of tomatoes in U.S. production areas, either with plants of the same species or with plants of different species, appears to be an extremely rare event, if it happens at all. Consequently, there will not be any significant outcrossing of tomato crops containing the *kan*^{r} gene.

Weediness potential of tomato. The weediness potential of transformed tomato is very low due to the characteristics of the crop and the inserted gene: tomato is not a weed in the U.S., the *kan*^{r} gene has no effect on crop fitness nor provides any selective advantage, field trials with transgenic tomato have no changes in weediness potential (Appendix EA-16). No members of the *Lycopersicon* genus, cultivated or wild, are considered weedy (Weed Science Society of America 1989). Other favorable characteristics have already been bred into tomato using classical breeding techniques and there has been no history of weediness problems.

G.1.1.2 Cotton

Cross-pollination of cotton with same species. Cotton is normally considered to be a self-pollinating crop, although natural outcrossing may range from zero to more than 50% (Simpson 1954). The cotton

floral structure provides direct contact of the anthers with the stigma (Bridge 1980). Since pollen shed occurs when the stigma is receptive, self-pollination takes place readily during the single day of anthesis and self-fertilization follows. To effect cross-pollination, foreign pollen must successfully compete with the 35,000 pollen grains produced by the average cotton flower (Vaissiere 1990). Cotton pollen is not transferred by wind due to its heavy, sticky nature (Niles and Feaster 1984). Natural cross-pollination results from pollen being carried by insects, bees being the most important cotton pollinators. Populations of pollen-carrying insects are influenced by proximity of other vegetation, climate and amount of insecticide spraying in the vicinity. Under U.S. agricultural production practices, insecticide applications keep pollinator populations at very low levels.

Further evidence for the limited amount of cross-pollination that occurs in cotton comes from isolation distances established for certification of hybrid plant materials by the AOSCA Handbook (1971). For cotton, the required isolation distance from any contaminating source is 100 feet for both foundation and certified seed.

Calgene has used hand-pollination techniques to cross transgenic cotton lines containing the *kan*^{r} gene with non-transgenic cotton lines to produce hybrids that contain the *kan*^{r} gene. Such breeding practices are common for cotton variety development and it is expected that specific genes, such as the *kan*^{r} gene, could be transferred to other cotton lines.

Regulated field trials planted with non-transgenic borders have been conducted by Calgene using cotton lines containing the *kan*^{r} gene. The covalently linked BXN gene was used as a convenient marker to determine the frequency of pollination of the border plants with transgenic pollen.

This study (Kareiva and Morris 1993) along with other published information on transgenic cotton (Umbeck et al. 1991) provides an information base to compare outcrossing rates between *kan*^{r} -containing cotton and non-transgenic varieties (Vaissiere 1990). There is agreement between the three studies, which were conducted at different locations using different genetic markers. All three had the same conclusion: that pollen carryover decreased very rapidly as the distance decreased and that there was very little pollen transfer beyond 12 m (Kareiva and Morris 1993; and Umbeck et al. 1991). These data provide evidence that the distance of pollen movement is unchanged in transgenic lines containing the *kan*^{r} gene.

When considering outcrossing results at the same location (Stoneville, MS), two reports showed the same outcrossing frequency. Meredith and Bridge (1973) detected no outcrossing between adjacent plants in a study conducted in Stoneville, MS (the approximate limit of detection for the sample size and methods was approximately 0.046%). This was consistent with results obtained for BXN cotton (see Table 1 in Kareiva and Morris 1993), which showed no outcrossing in 4144 seeds at any of the 5 distances sampled.

These results support the conclusion that integration and expression of the *kan*^{r} gene did not alter outcrossing behavior of transgenic as compared to non-transgenic cotton.

Cross-pollination of cotton with wild relatives. Crossing of cultivated varieties of cotton in the U.S. with related wild species will be an extremely rare event. The cottons cultivated in the U.S. belong to two tetraploid species, either American Upland (*Gossypium hirsutum*) or Pima (*G. barbadense*). *G. hirsutum* originated in southern Mexico and Central America as a perennial shrub, but is grown as an annual in the U.S. (Poehlman 1988). Related wild species in the continental U.S. are diploid, such as *Gossypium thurberi* which occurs in Arizona. Cultivated tetraploid varieties do not readily cross with diploid species due to the difference in ploidy level. Diploid cotton species have been crossed with tetraploids in breeding programs, but the vigor of the hybrid seed is low and hybrid plants are usually infertile (Munro 1987).

One native tetraploid *Gossypium* species is found in the Hawaiian islands. *G. tomentosum*, which occurs only in Hawaii (Fryxell 1984), will cross with *G. hirsutum*; however, the hybrids suffer from poor fertility and low vigor which reduces persistence relative to the native species. *G. tomentosum* was once considered to have weedy tendencies, but it is not generally considered a weed pest (Crockett 1977, Fryxell 1979, Holm et al. 1977, State of California 1990). In fact it is now considered a vulnerable native plant due to loss of habitat.

There will not be significant outcrossing of cultivated cotton containing the *kan*^{r} gene with wild relatives.

Weediness potential of cotton. The weediness potential of transformed cotton is very low due to the characteristics of the crop, the characteristics of the transformed gene and normal agricultural practices. Field trials with transgenic cotton have demonstrated no changes in weediness potential (Appendix EA-16). The members of the *Gossypium* genus, cultivated and wild species, are not considered weedy (Crockett 1977, Fryxell 1979, Holm et al. 1977, State of California 1990). Also, as stated above, no tetraploid wild species are known to be

Outcrossing rates measured within field plots of cultivated *B. napus* vary, but average 20-30% (Downey 1992; Huhn and Rakow 1979; Rakow and Woods 1987; see also review in Bing 1991). Outcrossing to neighboring areas has been more difficult to measure, but one study reported a frequency of 5-15% outcrossing to an adjacent plot (Huhn and Rakow 1979). Outcrossing to greater distances using a strain of *B. napus* with a recessive mutation as the pollen recipient and commercial variety of *B. napus* as the pollen donor has been reported in Downey and Bing (1990) and in Downey (1992). Recipient blocks were 46m square in size and placed 46, 137 and 366m away from a field planting of *B. napus* and the outcrossing rates were 2.1, 1.1 and 0.6% respectively. The scale of this study provides outcrossing rates that can be expected from commercial plantings of *B. napus*.

Outcrossing rates have been measured using *B. napus* genetically engineered for herbicide tolerance as the pollen parent and nontransformed plants as pollen recipients. Since the herbicide tolerance trait is expressed throughout the plant life cycle and is dominant, large numbers of progeny can be reliably screened at the seedling stage. Data, such as those in presented in Appendix EA-18 (Tables 1, 2 and 3), confirm that outcrossing rates fall off with distance and are generally quite low, although still measurable at distances > 50 m.

Outcrossing rates obtained from Calgene's oil-modified canola field plots in two locations in the U.S. show a similar trend, as distance from the transgenic plots increases, pollen transfer and successful pollination drops. Outcrossing rate was measured using a seed germination assay to detect resistance to the antibiotic, kanamycin (Morris et al. 1993). A description of these studies is included in Appendix EA-20, Issue 6.

Outcrossing rates for transgenic *B. napus* measured in the U.S. are comparable to those measured in the U.K., Canada and France. Of even greater relevance to this document, (although we do not have direct comparisons from controlled experiments, but only comparisons from limited literature values) Calgene's and other transgenic *B. napus* do not exhibit greater rates of outcrossing than nontransgenic lines. Data summaries for all outcrossing studies cited are included in Appendix EA-18.

Cross-pollination of rapeseed with wild relatives. The *kan*^{r} gene could potentially move into wild or naturalized species by production of hybrid progeny from crosses with the oil-modified canola as either the pollen or maternal parent. However, if the oil-modified canola were the maternal parent, the vast majority of the seed would be rendered

indigenous to the areas in which the transformed crop plant will be grown. Furthermore, if cross-pollination did occur, the hybrids would be infertile, low in vigor (Munro 1987) and the antibiotic resistance trait would not provide any selective advantage. Cotton is not allowed to overwinter; therefore, there is little risk that the transformed gene will persist in the field or that the crop will revert to a naturalized state. Other favorable characteristics such as improved agronomic performance have already been bred into cotton using classical breeding techniques and there has been no history of weediness problems.

Additionally, cotton would be a very poor competitor in most of the Southern and Southeastern U.S. cotton-growing areas. In areas where freezing conditions occur, the cotton plant cannot overwinter and there is essentially no volunteerism from seed. The various species of the genus *Gossypium* typically occur in relatively arid parts of the tropics and subtropics. The genus contains only one member, *G. tomentosum,* which has been considered by some to have weedy tendencies, but it is not generally considered a weed. It is found only in the U.S. (Holm 1979), occurring in the Hawaiian archipelago (Fryxell 1984). Because cotton has no weedy relatives, the possibility that a cross between genetically engineered cotton and wild cotton is extremely unlikely and therefore not an environmental concern. The special case of *G. hirsutum* and *G. tomentosum* is also unlikely to increase weediness because of low vigor of the hybrid and lack of significant weediness of *G. tomentosum.*

G.1.1.3 Rapeseed

Cross-pollination of rapeseed with same species. Like tomatoes and cotton, rapeseed (*Brassica napus*) is also predominantly self-pollinating. Under field conditions, outcrossing rates depend on proximity of sexually compatible plants, the availability of insect pollinators (i.e., honey, leaf-cutter and bumble bees), weather and the genotype of the crop. High plant populations and the physical contact of racemes in full bloom account for much of the natural cross-pollination observed within adjacent field plantings. Wind is not an effective rapeseed pollinator and has impact in pollination mainly for enhancing self-pollination. Thus, the role of wind in cross pollination is in causing direct physical contact between adjacent plants, not in the movement of free pollen. Cross-pollination at greater distances is primarily dependent on insects, as the pollen is fairly heavy and sticky and cannot travel more than a few yards without insect pollinators (Downey and Röbbelen 1989). Successful pollination is also dependent upon pollen viability, which decreases rapidly with time.

nonviable by crushing for oil production after harvest (Relatively small numbers of seed would also be disseminated along roadsides during transport from the field to the crushing plant. In most production areas such volunteers are controlled with mowing or herbicide application.). Hybrid seed that escaped harvest and remained in the field could germinate the following year (dormancy in such F1 seed would be 5% or less), but would likely be controlled by cultivation and/or herbicide application to the new (non-canola) crop. Thus, the most likely path for movement of a gene into naturalized species would be through pollen from the oil-modified canola, followed by backcrossing to the naturalized species in areas outside of cultivated fields.

After a thorough review of the literature, we have concluded that only three species are of concern as potential pollen recipients from the genetically modified *B. napus* plants under field conditions in the U.S. and Canada: *B. napus, B. rapa* and *B. juncea* (Bing 1991; Bing et al. 1991; Downey and Bing 1990; Downey 1992; Kerlan et al. 1991; Salam and Downey 1978; Appendix EA-15) Of these, successful crosses are most likely with cultivated *B. napus*, and the likelihood of these crosses can be significantly reduced with proper agronomic practices. For a summary of the literature review, see Appendix EA-18 (Table 4).

Recent studies using controlled and natural pollination conditions in western Canada (Saskatchewan) for rapeseed and its weedy relatives (*B. nigra* and *Sinapis arvensis* syn. *B. kaber*) have concluded that the natural barriers for gene flow into the weedy relatives are formidable and that gene flow would not occur (Bing et al. 1991). The PROSAMO group has also studied rapeseed and its wild relatives with similar conclusions: hybrids are not made under a variety of field conditions in the U.K. and laboratory produced hybrids are sterile in the field (Cherfas 1991). Recent studies in France with additional wild relatives (*Hirschfeldia incana* syn. *B. adpressa* and *Raphanus raphanistrum*) show that field crosses can occur under very unusual circumstances but are extremely unlikely (Baranger et al. 1992; Kerlan et al. 1992; Chevre et al. 1992).

Crosses with other *B. napus*. *Brassica napus* canola readily crosses with other types of cultivated *B. napus*, including other rapeseed, fodder rape and rutabaga. The probability of gene introgression from oil-modified *B. napus* into fodder rape or rutabaga is very low. Both are very minor crops in North America and are harvested before seed is set. Gene introgression into rapeseed can be deterred using standard agronomic practices for production of that crop. For example, it has always been necessary to isolate canola fields from fields of high erucic acid rapeseed (HEAR) to prevent the canola from producing higher

than acceptable levels of erucic acid after receiving pollen from a HEAR cultivar.

Crosses with *B. juncea*. *B. napus* is capable of acting as the pollen donor in crosses with *B. juncea*, cultivated as Indian or brown mustard although fertility of the hybrids is low (Bing 1991; Dhillon et al. 1985; Heyn 1977; Roy 1980; Appendix EA-18, Table 9). Most pollinations yield few if any seed (0-7), although some genotype combinations are highly fertile (Heyn 1977; Roy 1980). Viability of pollen from hybrid plants is less than 10% (Bing 1991). Under field conditions in western Canada with *B. napus* and *B. juncea* interplanted, an average of 4 hybrid seed per plant (4.7% of seeds tested) were produced on the maternal *B. juncea* plants. Many of these F1 plants were completely infertile and produced no seed, 50% produced only 5 seed, 10% produced up to 25 seed and the remainder produced intermediate amounts of seed (6 to 15 seed per plant) under open pollinating conditions in a greenhouse (Bing 1991). Using herbicide tolerant *B. napus* as the pollen parent, 0.3% and 0.1% of seed were hybrid in two years of field trials. Fertility of the hybrids was very low, but actual values were not given (Bing 1991).

Hybrids between *B. napus* and *B. juncea* have been generated in field experiments, but we did not find any published reports of natural field hybrids being formed. The distribution of naturalized *B. juncea* is sparse although widespread throughout temperate North America.

Crosses with *B. rapa*. *B. napus* and cultivated *B. rapa* can be interfertile and spontaneous crosses have been observed where the two species have been grown in adjacent cultivation (Downey 1992). In experimental field trials, these crosses are most successful with *B. napus* as the pollen recipient, which gives *B. napus* canola growers quite an incentive to ensure isolation, especially for a niche market or specialty crop. Many *B. rapa* crops, such as turnips, fodder rape and chinese cabbage, are harvested before seed is set in production fields, so that the likelihood of gene introgression from High Stearate *B. napus* into any of these crops is negligible, and crossing into *B. rapa* rapeseed is the only concern. With *B. napus* as the pollen parent in *B. rapa* crosses, an average of 9 viable seed were produced per flower pollinated under greenhouse conditions and the majority of these were sterile (Bing 1991). Under field conditions with *B. napus* and *B. rapa* interplanted, 1.3% of seeds produced on the maternal *B. rapa* plants were hybrid. These F1 plants were relatively infertile when grown under open pollination conditions (Bing 1991). Rapeseed seed production is commonly carried out using isolation conditions (e.g. AOSCA standards). Thus, with proper agronomic practices (especially crop rotation and field separation), introgression of genetic material

into cultivated *B. rapa* from the High Stearate *B. napus* should be extremely rare.

Since wild or naturalized *B. rapa* is common and widespread throughout temperate North America, and occurs in cultivated and disturbed areas, it is much more likely than either *B. juncea* or cultivated *B. rapa* to be in close enough physical proximity to receive pollen from cultivated *B. napus*. Natural field hybrids between *B. napus* and *B. rapa* are often identified in western Canada (Downey 1992).

In studies conducted by Calgene crossing oil-modified rapeseed pollen onto both cultivated and wild *B. rapa*, we found that the vast majority of pollinations do not result in the formation of viable seed of a crop/weed hybrid. Oil-modified rapeseed was not more able than the parent cultivar to act as pollen parent and produce seed on *B. rapa*. Crop/weed hybrid seed had poor fertility and high seedling mortality. Study results are presented in Appendix EA-20, Issues 7 and 8.

Crosses with *B. nigra* Under field conditions, hybrids were either not produced at all (Baranger et al. 1992) or were produced in very low numbers and were male sterile (Bing 1991).

Crosses with *Sinapis arvensis* Under field conditions, hybrids were unable to be formed (Baranger et al. 1992; Bing 1991).

Crosses with *Raphanus raphanistrum* Studies are still underway to determine whether hybrids are formed under field conditions when *R. raphanistrum* is the pollen recipient (Baranger et al. 1992). *R. raphanistrum* was able to produce hybrid seed on male sterile *B. napus* containing a *Raphanus* cytoplasm under field conditions when the two types of plants were adjacent and planted in equal numbers (Baranger et al. 1992). These hybrids were barely able to self and produced fewer than 1 seed per plant, indicating that they would not persist. Laboratory produced hybrids (manual pollination and fertilized ovary culture) were produced in reciprocal crosses between Basta tolerant *B. napus* and wild *R. raphanistrum*. When these F1 hybrids were backcrossed to *R. raphanistrum*, progeny were only produced on plants containing the *B. napus* cytoplasm, and at a very low frequency (0.004 hybrids per manual pollination). When the BC1 progeny were pollinated by *R. raphanistrum*, numerous progeny were produced, but all had lost both the *B. napus* phenotypic traits and Basta tolerance, indicating that most or all of the *B. napus* genome had been lost. We conclude from these studies that under normal conditions of canola cultivation few or no hybrids would ever be formed between *B. napus* and *R. raphanistrum*, and that such hybrids would neither persist nor

pass the *B. napus* genetic material into wild *Raphanus* populations.

Crosses with *B. adpressa* (syn. *Hirschfeldia incana*) Manual crosses made with *B. adpressa* as either parent yield progeny that are exclusively male sterile and almost entirely female sterile as well (Baranger et al. 1992; Kerlan et al. 1992). Laboratory produced hybrids containing *B. adpressa* cytoplasm were unable to backcross to *B. adpressa* (Baranger et al. 1992). Under field conditions, very low numbers of hybrids (ca. 5 seed per plant) have been produced on male sterile *B. napus* interplanted with equal numbers of *B. adpressa* (Lefol et al. 1991; Darmency and Renard 1992; Baranger et al. 1992; Chevre et al. 1992). Field produced hybrids are predominantly sterile (Baranger et al. 1992). The probability of such hybrids being formed is vanishingly small when *B. napus* pollen is present and weed infestation is low to moderate. As far as we have been able to determine, *B. adpressa* does not occur east of Nevada.

Fate of rapeseed hybrid progeny. F1 hybrids formed between either *B juncea* or *B. rapa* and *B. napus* have an intermediate number of chromosomes. In subsequent generations, the most likely scenario is for the hybrids to revert to a chromosome number close to one of the parents. Fertility of individual hybrid plants is a function of chromosome number. Salam and Downey (1978) found that a disproportionate number of F2 hybrids formed between *B. rapa* and *B. napus* had chromosome numbers close to *B. napus* and that these plants were more fertile than F2 plants with lower chromosome numbers (more like *B. rapa*), Appendix EA-18, Figure 2. Further, F1 plants backcrossed to *B. rapa* were still highly infertile and exhibited high (25-33 %) seedling mortality. Under field conditions, in the absence of an introduced selective advantage and proper selection pressure, the hybrids most likely to persist are those with the greatest seed output, and these will be the ones with genotypes most like *B. napus*. The phenomenon of hybrids reverting to cultivated forms was also observed by Bing (1991).

With *B. napus* as the pollen parent, F1 hybrid plants exhibit < 10% fertility in *B. rapa* crosses (Bing 1991), and both F2 plants and backcrossed F1 plants exhibited both low fertility and considerable seedling mortality (Salam and Downey 1978). Thus, the persistence of progeny from such a cross may still be considered rather unlikely unless they can backcross into *B. napus*. Backcross progeny would quickly reconstitute a *B. napus* form and thus not be weedy like *B. rapa*.

The compatibility of *B. juncea*/*B. napus* varies from 0 to 100%, depending upon the individual genotypes (Roy 1980; Sacristan and

Gerdemann 1986; Bing 1991). However, regardless of the ease with which hybrids are formed initially, most F1 progeny from *B. juncea/B. napus* crosses are either sterile or only slightly fertile when selfed or open pollinated (Dhillon et al. 1985; Roy 1980). F1 plants also display abnormal root morphology (Dhillon et al. 1985; Sacristan and Gerdemann 1986; Roy 1978). F2 plants retain a high degree of infertility or are sterile. F1s backcrossed to *B. napus* were still primarily (89%) infertile or partially fertile, producing 0-4 seed per pollination, although a few were highly fertile (the reciprocal backcross was not performed), (Roy 1980). Hybrids formed with *B. juncea* that possessed reasonable fertility were reported to preferentially revert to the *B. napus* form by the F2 generation (Roy 1980).

Under natural (non-experimental) field conditions, hybrids between *B. napus* and *B. juncea* have not been reported. These should be much rarer than *B. napus/B. rapa* hybrids, not only because *B. juncea* is so much less prevalent, but also because self-pollination in *B. juncea* normally occurs as the flower opens or very shortly thereafter, whereas *B. rapa* is self-incompatible.

As discussed above, hybrids between *B. napus* and *B. adpressa, B. nigra, Sinapis arvensis* or *Raphanus raphanistrum* are either not formed, or are sterile, or insufficiently fertile to maintain themselves by selfing, and/or are unable to backcross to their weedy parent or lose the *B. napus* genome during backcrossing. Thus, we view the potential to hybridize with these weedy species as not posing any concern and do not discuss them further.

Persistence of the transgene in naturalized rapeseed hybrids or wild relatives. Persistence of naturalized hybrids, especially in a perennial community, will be dependent upon sufficient seed output, ability to compete and ability to form seed banks in the soil. We have already discussed above the relative infertility of F1 and F2 hybrids formed with *B. rapa* and their greater seedling mortality. Introgression of the gene into persistent populations of *B. rapa* through backcrossing is also unlikely, given the infertility of such backcross progeny (Salam and Downey 1978). Formation of seed banks from such hybrids will also be unlikely, due to low seed output of hybrids and backcross progeny. Hybrids show intermediate levels of dormancy when compared to the cultivated and wild parents (Adler et al. 1993) and are strongly influenced by the dormancy tendencies of the maternal parent, i.e., when *B. rapa* is the maternal parent, hybrids show significantly more dormancy than when *B. napus* is the maternal parent and both types of hybrids exhibit significantly less seed dormancy than naturalized forms of *B. rapa* (Adler et al. 1993; Linder and Schmitt 1993). The combination of lower fertility and lower seed dormancy reduce the

potential to form persistent seed banks and thus, make it unlikely that hybrid forms would persist in the environment.

The situation with *B. juncea* hybrids is somewhat different. Hybrids are quite infertile as discussed above. In addition, since naturalized *B. juncea* does not exhibit levels of seed dormancy normally associated with weeds (Downey 1992, personal communication) hybrids would not be likely to form large seed banks even if they were highly fertile.

The tendency for hybrids to revert to a *B. napus* form upon selfing further lessens the probability that persistent populations of weedy genotypes containing the *kan*^{r} gene will be formed.

Potential transfer to plant species with which *B. napus* cannot interbreed. The only potential mechanism for transfer of genetic material to plant species with which *B. napus* cannot interbreed would be through "bridging" (bridging is defined as "a mating made to transfer one or more genes between two reproductively isolated species by first transferring them to an intermediate species that is sexually compatible with the other two species" [King and Stansfield 1985]). For example, within the genus *Brassica*, if a hybrid were formed between the oil-modified *B. napus* and *B. juncea*, could the hybrid then cross into the weedy species *B. nigra*, since *B. juncea* and *B. nigra* share the bb genome? We discuss this potential below. A brief background discussion of the various diploid and amphidiploid genomes present in related *Brassica* species is provided in Appendix EA-18.

As we discussed, formation of such hybrids (e.g. *B. napus / B. juncea* hybrids) will be a rare event and persistence of hybrid individuals is extremely unlikely due to poor fertility, lack of any selective advantage of the introduced gene, high seedling mortality and poor germination. Further, the cross *B. juncea* X *B. nigra* is not fully compatible and the cross between a *B. napus / B. juncea* hybrid and *B. nigra* should be even less compatible. Finally, since *B. napus* and *B. nigra* do not share a genome (Appendix EA-18, Figure 1), the introduced gene would have to be transferred via chromosomal crossing over to the bb genome in the *B. napus / B. juncea* hybrid in order to be stably introduced into *B. nigra*. Thus, we deem the probability for transfer of the introduced genetic construct from *B. napus* to the closely related but not sexually compatible weedy species *B. nigra* via a bridging mechanism to be essentially zero. Probability of transfer of the introduced genetic construct to even less closely related species is also essentially zero. Bing (1991) also concluded that "under the field environment of western Canada natural gene transfer from *B. napus* to *S. arvensis* and *B. nigra*, either directly or indirectly through bridging via *B. campestris* or *B. juncea*, would not occur."

Weediness potential in rapeseed. *B. napus* is not a weedy pest. *Brassica napus* is not listed as a weed by the Weed Science Society of America (1989) or in the 1992 Weed Control Manual. *Brassica napus* is the only naturalized *Brassica* that is not noted as a pestiferous weed by Rollins (1981). *B. napus* is not listed in *Weeds of the United States* (Lorenzi and Jeffrey 1987). No *Brassica* is noxious (Federal Noxious Weed Regulation, 7 CFR 360) and none of the *Brassica* species are listed in *The World's Worst Weeds* (Holm 1977).

All of the *Brassica* species currently present in North America have been introduced (Table G.1.1.3-1). Those species that are weedy either escaped from cultivation or were introduced into fields as seed contaminants. *B. napus* has a slower relative growth rate than either *B. juncea* or *B. rapa* (Tsunoda 1980), which may reduce its competitive ability and thus its weediness potential, in spite of high seed production. Half of the *B. napus* genome is derived from *B. oleracea*, an extremely slow growing species that has never naturalized. *B. juncea*, on the other hand, is derived from *B. nigra* and *B. rapa*, both of which are weedy.

Table G.1.1.3-1. Members of the genus *Brassica* found naturalized in North America. Those denoted by * are considered pestiferous weeds by Rollins (1981). Those denoted by # are considered weeds by Lorenzi and Jeffrey (1987).

Brassica species	common name	distribution
*#*B. rapa*	field mustard bird's rape	common and widespread throughout temperate North America, occurs in cultivated and disturbed areas
**B. elongata*	none	roadside weed of eastern Nevada
* *B. hirta* syn *Sinapis alba*	white mustard	widespread but sporadic in North America, abundant in some localities
**B. juncea*	Chinese Mustard Indian Mustard	sparse, but widespread throughout temperate North America, occurs in cultivated and disturbed areas
*#*B. kaber* syn. *Sinapis arvensis*	wild mustard charlock	abundant throughout the temperate agricultural areas of North America, especially in newly disturbed areas
B. napus	rape	sporadic in temperate North America, waste places
*#*B. nigra*	black mustard	widespread in temperate North America, especially common the Central Valley of California, sporadic in the more northerly areas of the continent
**B. tournefortii*	wild turnip	roadsides and old fields of the Southwest

Tsunoda (1980) reports that without favorable, intensive cultivation, domesticated forms of *B. napus* cannot compete with common crop plants and that its naturalized forms are quite distinct from domesticated forms. In the U.S. and Canada, naturalized forms of *B. napus* are sporadically distributed. In the U.K., naturalized forms of *B. napus* are more widespread (Mitchell-Olds 1992) and can survive for several years. Repeated introduction of *B. napus* may have been

important in establishing records of its occurrence outside of cultivation, rather than one or a few escape events forming the basis of self-perpetuating naturalized populations (van der Meijden and de Vries 1992). In the absence of clarifying data, for the sake of this risk assessment it should be assumed that *B. napus* has the potential to become more widely naturalized in the U.S. and Canada.

B. napus can persist in cultivated fields because it produces large numbers of small seeds (some of which may spill before or during harvest) with some potential for dormancy and overwintering. In areas with a rapeseed growing history (such as Canada) volunteer rapeseed is an issue for farmers when crop rotation practices are not followed and a new rapeseed variety is introduced to a field that had rapeseed the previous year. *B. napus* is easily controlled in other crops since it is sensitive to substituted ureas, norflurazon, triazines, imidazolinones, most sulfonylureas and other classes of herbicides. Not surprisingly, there are no herbicides selective enough to cull volunteer rapeseed from a new crop of rapeseed. Volunteer rapeseed can be controlled using agronomic practices such as 1) rotation of fields out of rapeseed and concomitant use of selective herbicides or 2) field cultivation after germination of seed.

Whether in managed, agricultural settings or in natural, non-managed areas, the characteristics that may lead to weediness potential are the same. Calgene used the issues identified by Tiedje et al. (1989) as a basis for its evaluation. For rapeseed, an evaluation of the weediness, invasiveness, persistence and selective advantage of the *kan*^{r} and oil-modified trait was made using both literature and studies conducted with oil-modified rapeseed. Data generated to support the conclusion that transgenic rapeseed containing the *kan*^{r} gene and genes for oil-modification is no more weedy than its parent is presented in Appendix EA-20.

Field studies have been completed to evaluate transgenic *B. napus* rapeseed in Europe and in North America. Calgene has completed six traditional plant breeding (replicated) trials under USDA permitted containment and data summary comparing the performance of the transgenic lines to that of the parent is provided in Appendix EA-20, Issue 1. Specific field studies designed to evaluate aspects of weediness potential have been completed by the PROSAMO group in the UK (Crawley 1992.). In collaboration with Calgene, Brown University has completed buried seed persistence studies at two field locations in the U.S. (Appendix EA-20, Issue 3).

In the PROSAMO studies conducted to evaluate persistence potential, buried seed from transgenic oilseed lines consistently had greater

mortality than the nontransgenic lines, and exposed seed were quickly eaten by small predators (Crawley 1992.). In field studies with buried seed in Georgia and California, the oil-modified rapeseed was not more persistent than the parent and loss of seed from the seed bank was quite rapid, half-life in 0.2-1.5 months (Appendix EA-20, Issue 3). Both studies compared the parent canola variety, with rapeseed containing the *kan*^{r} gene and support a conclusion that kanamycin resistant rapeseed will not persist for long periods in the environment

The invasive potential of transgenic *B. napus* has been evaluated under field conditions in the UK (Cherfas 1991) and it was found to be non invasive. Preliminary data indicated "that these plants do not outgrow their competitors in the wild, nor is there any evidence that they pass on their foreign genes to other species." In these studies Westar, a widely grown canola variety, is compared to Westar engineered for resistance to the herbicide, Basta (syn. Ignite), or the antibiotic, kanamycin. Further results, reported at Goslar, supported the initial assessment (Scheffler et al. 1992; Crawley 1992).

Transgenic oilseed rape was unable to become established and produce seed when sown to undisturbed vegetation in any of twelve experimental sites in the UK. over a two year period (Crawley 1992.) Further, even in fertile, disturbed soil the transgenic rape had a net reproductive rate less than 1, meaning that it would become extinct without human intervention (Crawley 1992).

In addition, evaluation of invasiveness and persistence with Calgene's oil-modified rapeseed lines were completed in laboratory studies at the University of Georgia and Brown University (Appendix EA-20, Issue 2,3; Rao and Raymer 1993 a,b,c; Linder and Schmitt 1993). Seed germination, dormancy and seedling vigor were studied under a range of environments. Given the experimental conditions, no evidence was gathered to suggest that Calgene's *kan*^{r}, oil-modified rapeseed is more likely to establish than the parental type.

Experiments conducted by Calgene provide support for the views widely held by qualified experts that neither *kan*^{r} nor oil modification genes confer a selective advantage to *B. napus*. Replicated field trials were conducted in California, Georgia and Michigan with the oil-modified rapeseed lines. Traits such as seed germination, seed yield, seedling vigor etc. were measured in the parental line, other cultivars and numerous transgenic lines. The agronomic performance parameters measured for the transgenic lines were not outside the range of expected values based on the history of the crop (Appendix EA-20, Issues 1,4, 5).

G.1.2 Plant-to-Bacteria Transfer: Natural Transformation

Potential transfer to microorganisms. Concerns have been expressed about potential transfer of DNA from transgenic plants to microorganisms. To date, examples of such horizontal gene flow are very few and difficult, if not impossible, to prove.

The probability of horizontal gene flow is vanishingly small for the following reasons (IFBC 1990):

1. The introduced genes are permanently incorporated into the plant chromosome.
2. Transgenic plants do not contain genes required for transport of DNA into bacteria.
3. If bacteria acquired the DNA through passive uptake from decomposed plant tissue, the bacterial cell could not "read" the genes since they do not have bacterial promoters.

In addition, no mechanism for transfer of genes from plants to microorganisms is known and no cases of such transfer have been adequately documented. Carlson and Chelm (1986) argued for an eukaryotic (plant) origin of glutamine synthetase II in bacteria, albeit over an evolutionary time period. They suggested that this was evidence that horizontal gene flow from plants to microorganisms had occurred at one point in evolution. However, their paper was directly refuted by Shatters and Kahn (1989) who concluded that "the GS [glutamine synthase] proteins are highly conserved and the divergence of these proteins is proportional to the phylogenetic divergence of the organisms from which the sequences were determined. No transfer of genes across large taxonomic gaps is needed to explain the presence of GSII in these bacteria." Other "evidence" that horizontal gene flow occurs from plants to microorganisms involves transient changes (non-heritable) such as transencapsidation of chloroplast DNA (Rochon and Siegel 1984) or possibly endocytosis (Bryngelsson et al. 1988), neither of which have been shown to result in actual transfer of genes from plants to microorganisms. No mechanism by which plant DNA could be incorporated from plants into the genomes of the microorganisms has been proposed. For *Agrobacterium*-mediated transformation, Zambryski et al. (1982) provide evidence that once inserted DNA is integrated into the plant host genome, it cannot be remobilized even if acted on again by *vir* genes.

Recently, Smith et al. (1992) presented arguments for the occurrence of horizontal gene transfer. Two cases of transfer from eukaryotes to prokaryotes, and two cases of transfer from prokaryotes to eukaryotes were considered by the authors as likely to have occurred over an evolutionary time period. If their assertions are true (and these authors admit that

"horizontal gene transfers are difficult to prove" and that additional data "may also lead us to reconsider some of the cases that at present appear to be sound"), these occurrences can still be considered to be extremely rare.

Even if we were to assume that transfer of the *kan*^{r} gene to bacteria could occur easily, the consequences of such transfer would be insignificant. There would be no significant increase in the numbers of resistant bacteria present relative to background numbers: the worst case scenario predicts that background levels of kanamycin resistant organisms would be 7 orders of magnitude greater than the numbers of organisms resistant through incorporation of the *kan*^{r} gene from plant tissues (Appendix EA-9).

Probability of transferring the *kan*^{r} gene to bacteria. This section of the EA evaluates the probability of transferring *kan*^{r} genes from the crop plants containing the genes to bacteria in the soil that do not contain the genes. Bacteria were selected as the recipient organisms because of their importance in both human and natural ecosystems and also because information about the mechanism and frequency of natural transformation of certain genera is available.

The only plausible mechanism by which gene transfer could occur between plants and bacteria is through natural transformation (See Appendix EA-13 on Natural Transformation). For this EA, we have considered the following scenario. Free plant DNA containing an intact *kan*^{r} gene becomes disassociated from a plant cell and exists long enough in the soil to be taken up by some soil bacterium, incorporated into the bacterial genome and expressed, although at a relatively low frequency. Using worst-case assumptions, the frequency with which this might occur and the resultant number of transformants is compared to the prevalence of naturally-occurring, kanamycin-resistant organisms.

Evidence suggests that bacteria in the environment can take up free DNA (Stotzky 1989). There are also data to suggest that appreciable quantities of free DNA exist in the environment, especially in marine ecosystems (Stotzky 1989). However, naked DNA in the environment is also considered to be highly susceptible to degradation, especially in soil. In addition, DNA may adsorb to soil particles and become sequestered, unavailable for uptake by soil microbes. These factors may render natural transformation a relatively unimportant mechanism of genetic transfer in soil (Stotzky 1989).

In its "Interpretive Ruling on Calgene, Inc., Petition for Determination of Regulatory Status of FLAVR SAVR™ Tomato," the USDA APHIS concluded that it "has not identified any potential effects arising from any

rare gene transfer from FLAVR SAVR™ tomatoes to microorganisms, should any occur" (Appendix EA-11).

Total yearly release of *kan*^{r} DNA. In Appendix EA-9 and Section G.2.1, we have calculated and presented estimates of the total yearly release of APH(3')II (gene product) from agricultural residues. In this section, for the sake of completeness, we present estimates of the maximum total yearly release of *kan*^{r} DNA from agricultural residues. Based on the assumptions and calculations shown in Appendix EA-9, the following estimates of yearly *kan*^{r} DNA release were determined (see Table G-1 for total acres harvested in the U.S.):

- Tomato 2.2 kg/yr = 15.7 x µg/acre/yr
- Cotton 61.0 kg/yr = 4.9 x µg/acre/yr
- Rapeseed 4.4 kg/yr = 0.9 x µg/acre/yr
- Total 67.6 kg/yr = 3.8 x µg/acre/yr

These total *kan*^{r} DNA release estimates represent less than 8.8 x 10^{-9}% of the total crop biomass produced yearly for these crop plant species. The implications of DNA release, other than for natural transformation which is considered in the next section, would thus not be anticipated to be significant. Also, it must be remembered that these values are estimates of environmental "loading" at the field site, not estimates of actual exposure. DNA is subject to degradation and sequestering in the soil. In addition, the total yearly releases of *kan*^{r} DNA presented above represent gross overestimates of the true values due to the fact that the fruit or seed, which is usually harvested, is included in the field biomass values and due to the fact that not all the acreage of these crops is expected to be planted with transgenic varieties.

G.1.2.1 Natural Transformation and Agricultural Residues

Several bacterial genera found in soils are known to have natural transformation systems (Appendix EA-13 on Natural Transformation). In addition, large quantities of plant material (biomass) are left in the field after the harvest of a crop. Since the fate of the DNA (i.e., *kan*^{r} genes) in this field debris is not known, this section of the EA considers the possibility that DNA, or more specifically *kan*^{r} genes, released from plant debris left in the field are taken up and integrated by soil bacteria.

There are two different scenarios to be examined. In one, the expected frequency of transformation by plant DNA containing the *kan*^{r} gene is

considered for an organism capable of natural transformation in the field (e.g. *B. subtilis*), but which has no sequence homology to the incoming DNA. The second scenario involves transformation of *A. tumefaciens* which does not have an efficient natural transformation system, but does have sequence homology to the incoming DNA. The step-by-step calculations for each scenario are presented in Appendix EA-9. Summaries of the results and the assumptions used are discussed below.

The amount of DNA in the plant biomass can be used to calculate the concentration of DNA in the soil moisture. This calculation was done for tomato, cotton and rapeseed using estimates of biomass and the known DNA content for cells of each plant species. It should be noted that the biomass levels used are high. These levels represent the case where the crop was not harvested and the entire plant was plowed under as "green manure." A further assumption, in the case of cotton, was that no defoliant was used to treat the field.

The probability of transformation of soil microbes with DNA from plant debris left in the field can be expressed as the product of a number of factors which can be classified into four general areas.

1. Factors relating to DNA concentration:

   - Mass of plant DNA per hectare.
   - Fraction of plant DNA that is not degraded.
   - Fraction of plant DNA that is not sequestered on soil particles or is not in undegraded plant tissue (i.e., "free" DNA).
   - Total volume of soil moisture per hectare.

2. Factors relating to DNA uptake:

   - Fraction of organisms belonging to genera that can be transformed.
   - Fraction of organisms in transformable genera that are competent.
   - Frequency of natural transformation (if DNA in excess).
   - Correction factor for DNA concentration (if DNA not in excess).

[redacted]

- Correction factor for complexity of transforming genome.

3. Factors relating to size of transforming DNA (i.e., probability that DNA of a given length contains an intact *kan*^{r} gene).

4. Factors relating to recombination and expression:

   - Recombination frequency (homologous or illegitimate).
   - Probability that the gene is not disrupted by recombination.
   - Probability that the gene is expressed.
   - Probability that the gene product is active and stable.
   - Probability that the transformed cell is not already resistant to kanamycin or neomycin.

Most of the above factors will vary with soil conditions. The number of transformable microbes, number of competent microbes, the half-life of DNA in the soil, fraction of DNA that remains in undegraded plant tissue and moisture content of the soil will depend on the location and weather conditions, as well as agronomic practices employed.

We have estimated the probability of transformation using two different approaches. In the first approach, we have estimated the probability of transforming soil microbes by cascading worst-case assumptions, which gives an extremely unrealistic case (i.e., a worst, worst-case scenario). In the second approach, we have assumed a more likely case by making use of more realistic assumptions for most of the factors described above. The range produced by these two approaches can then be used to evaluate the risk that might be encountered through natural transformation.

Bacillus subtilis-type transformation. For the case of natural transformation and illegitimate recombination using *B. subtilis* as an example of a commonly occurring soil microbe which is highly transformable, estimates of the probability of producing kanamycin-resistant soil microbes range from 2×10^{-11} to 2.7×10^{-17} per year (Appendix EA-9).

Stotzky (1989) reports background levels of bacteria in soil to be 10^6 to

10^9 bacteria per gram of soil. If one assumes there are 10^7 viable bacteria per gram of soil (Atlas and Bartha 1987) and applies the probabilities of natural transformation estimated above, yearly transgenic agricultural residues result in from one transformant per 5 x 10^3 grams of soil per year to one transformant per 4 x 10^9 grams of soil per year, or alternatively, from about one to two transformants per hectare per year to 9 x 10^5 transformants per hectare per year.

This number should be compared to the background population of kanamycin-resistant microorganisms in the soil which, in one study, was about 1600 per gram of soil or about 7.2 x 10^{12} per hectare (Henschke and Schmidt 1989). This number could also be compared to the large fraction of *Rhizobium* strains resistant to kanamycin or neomycin (Young and Chao 1989). Thus, if any kanamycin-resistant bacteria are actually produced via natural transformation, they would represent an addition to the existing background population of, at most, 1.25 x 10^{-5}%, an infinitesimal amount.

In conclusion, it follows from the above calculations that at worst, kanamycin-resistant transformants resulting from plant DNA left in the fields would constitute not more than 1/10,000,000 of the existing kanamycin-resistant soil population. The most realistic estimate is that kanamycin-resistant bacteria resulting from transformation by plant DNA would represent an addition of about one organism to the total number of kanamycin-resistant soil microorganisms present in one hectare.

Agrobacterium tumefaciens-type transformation. The case of *A. tumefaciens* is considered separately since it represents a case in which there is no efficient natural transformation system, but certain portions of the T-DNA vector are homologous with the *Agrobacterium* Ti plasmid, which means one should allow for homologous recombination. The frequency of homologous recombination varies with the size and sequence of the homologous region, with estimates ranging from 10^{-2} to 10^{-5} (Comai *et al.* 1983). Others have reported combined transformation and homologous recombination frequencies, under optimal laboratory conditions, of 10^{-10} (Matzke and Chilton 1981).

If one uses the same approach as outlined above in the *B. subtilis* example, but assumes that the combined frequency of transformation and homologous recombination is 10^{-10}, the probabilities of transformation of *A. tumefaciens* would range from 2 x 10^{-14} to 1.3 x 10^{-21} per year. Assuming all bacteria in the soil are *A. tumefaciens* and that there are 10^7 viable bacteria per gram of soil, the number of

transformants that would be produced from plant DNA would range from 900 transformants per hectare to the more likely case of about one transformant per 18,000 hectares. If any kanamycin-resistant bacteria are actually produced through natural transformation under this scenario, they represent an addition to the existing background population of 1.25×10^{-8}%, an infinitesimal amount.

It follows from the above calculations that at worst, kanamycin-resistant transformants resulting from plant DNA left in the fields would constitute not more than 1/10,000,000,000 of the existing kanamycin-resistant soil population. The most realistic estimate is that kanamycin-resistant bacteria resulting from transformation by plant DNA would represent an addition of about one organism to the total number of kanamycin-resistant soil microorganisms present in 18,000 hectares.

G.1.2.2 Human, Animal and Processing Wastes: Natural Transformation

This pathway has already been dealt with indirectly in the previous section. In calculating the release of *kan*^{r} genes from agricultural residues, the crop biomass figures used included the weight of the entire plant. Thüs, any part of the plant subsequently removed for human or animal use, or during processing has already been accounted for in our calculations of environmental release from agricultural residues. Thus, no additional *kan*^{r} genes would enter the environment through human, animal and processing wastes.

The prerequisite for this particular pathway is that active, intact *kan*^{r} genes reach the environment after passing through one or a combination of the following: human digestive tract, animal digestive tract, or the typical steps used in processing of the respective product. The probability of active, intact *kan*^{r} genes surviving any of these three is essentially zero (Appendix EA-13, Redenbaugh et al. 1993).

G.1.2.3 Indigenous *Agrobacterium tumefaciens*: "Reverse Infection"

Another possible scenario under the bacteria-to-plant pathway is that *A. tumefaciens* naturally occurring in the environment could incorporate *kan*^{r} genes from crop plants containing the *kan*^{r} gene during *Agrobacterium* infection of the plant and subsequently transfer the gene to other plants during re-infection. This scenario is not likely as the evidence is that genes inserted via an *Agrobacterium*-mediated process are stably incorporated into the plant genome (Budar *et al.* 1986, Fillatti *et al.* 1987, Catlin *et al.* 1988, Deroles and Gardner 1988, and Umbeck *et al.* 1989).

Specifically, the transfer of genetic material from *A. tumefaciens* to a plant cell is strictly a one-way process which requires a large number of *vir* genes found on the Ti plasmid. These genes have not been found in plant cells and no evidence has yet been found to indicate that reverse transfer can or does occur. Therefore, *A. tumefaciens* naturally occurring in the environment should not be at risk for picking up *kan*^{r} genes from plants during infection or be at risk for transferring them to other plants.

G.1.3 Bacteria-to-Bacteria or Bacteria-to-Plant Transfer

If *A. tumefaciens* containing the *kan*^{r} gene were to escape into the environment, transfer of *kan*^{r} genes to other organisms could occur via two different pathways: (1) bacteria-to-bacteria (i.e., *A. tumefaciens* to other naturally-occurring bacteria), or (2) bacteria-to-plant (i.e., *A. tumefaciens* to other plants through the normal infection process). However, escape of *A. tumefaciens*, either via direct release or as a result of residual *A. tumefaciens* remaining associated with the transgenic plants, is not likely. These two escape scenarios are doubtful due to: (1) the implemented containment procedures discussed in Section F.2., (2) the crop plants containing the *kan*^{r} gene used in the field are several generations removed from the transgenic plants produced in the laboratory or greenhouse, and (3) any *Agrobacterium* associated with the transformed plants are killed before the plants leave the laboratory (transgenic plants from the laboratory are placed in a growth medium containing carbenicillin, cefotaxime, or other antibiotic, for a minimum of six weeks). Carbenicillin and cefotaxime are antibiotics that are highly effective in killing bacteria (i.e., residual *A. tumefaciens*), but will not harm the transgenic plant material.

Genetic transfer mechanisms available for bacteria-to-bacteria transfers can be grouped into three broad categories: conjugation, transduction and transformation. Conjugation (mating) involves DNA transfer via actual cell-to-cell contact between the recipient and the donor cell. Conjugation can involve either chromosomal genes or genes carried by plasmids, but is usually restricted to transfer of plasmids. Transduction involves the transfer of DNA from one cell to another via a bacterial virus or phage. Transformation is a process by which free DNA is inserted directly into a competent recipient cell. Natural transformation has already been discussed in relation to the plant-to-bacteria pathway and will not be discussed any further here. For a more detailed discussion of natural transformation, see Appendix EA-13.

In the bacteria-to-plant transfer scenario, an escaped *A. tumefaciens* strain

in which the normal T-DNA had been replaced by the *kan*^{r}-containing T-DNA (characterized in Appendix EA-1) would theoretically be capable of producing transgenic plant cells through the natural process of plant infection and T-DNA transfer. However, this transgenic *Agrobacterium* strain would not produce crown galls because laboratory strains are disarmed and nonpathogenic (Hood et al. 1986). Any transformed somatic cells would not produce a transgenic whole plant without human intervention, and the trait would thus not be heritable. Even if the *kan*^{r} gene were first transferred to a pathogenic (virulent) strain of *Agrobacterium* from the avirulent laboratory strain, and then transferred to a plant via infection of somatic plant cells, the *kan*^{r} gene would not be passed to any progeny. In both cases, the final result would be for such transformed cells to die when the parent plant dies.

G.1.4 Probability of Gene Expression

Even if *kan*^{r} genes were to be successfully incorporated into the genome of an organism by any of the mechanisms already discussed, there would still be the possibility that the genes would not be expressed (i.e., not produce gene product). In plants, the expression of stably incorporated antibiotic resistance genes appears to be highly variable. Fillatti *et al.* (1987) found that in glyphosate-tolerant tomatoes, the amount of protein (i.e., gene product) produced varied about 50-fold between plants derived from independent transformation events. Also, Deroles and Gardner (1988) found that over one-third of transgenic petunia plants that contained intact copies of a kanamycin resistance gene did not express their kanamycin resistance genes well. In addition, Jones *et al.* (1987) present data that, for *Agrobacterium*-mediated transformation in petunias, the probability of expression ranges from about 30% to 60%. Therefore, the low level of expression exhibited by antibiotic resistance genes incorporated into plants, in general, could reduce the potential impact of introducing crop plants containing antibiotic resistance genes into the environment.

For bacteria, the variability in expression of stably incorporated antibiotic resistance genes appears to be less well documented. Any variability in expression is highly dependent on the fate of antibiotic resistance genes within the bacterium (i.e., will antibiotic resistance genes be incorporated into genomic DNA or will they reside on plasmids?). The location within the bacterial genome of antibiotic resistance genes is also important in evaluating the ultimate fate of these genes in the environment.

G.2 Fate of APH(3')II

This section of the EA discusses the second potential concern of an environmental release: fate, transport and toxicity of the antibiotic resistance

gene product (APH(3')II). In this section, the amount of APH(3')II released into the environment and its concentration in soil are estimated using several "worst-case" assumptions. In addition, potential ecotoxicological effects from exposure to APH(3')II are discussed. This particular section of the EA is more analogous to a "traditional" EA than earlier sections. In this section, we are not dealing with a concept that is unique to biotechnology applications (i.e., gene transfer). We are concerned with the release and subsequent fate, transport and toxicity of a compound produced during the manufacture and use of a food processing aid.

The concern for the release of APH(3')II into the environment is considerably less than for the previous pathway which involved the probability of transfer of *kan*^{r} genes into other organisms in the environment. This reduced concern revolves around the fact that APH(3')II is a protein, just like similar proteins that are produced and released into the environment everyday.

There are several reasons why the levels of APH(3')II estimated to be released into the environment are of little concern. First, the estimated releases of APH(3')II calculated in this section make use of several "worst-case" assumptions and, in addition, the estimates presented are not estimates of exposure, but estimates of soil "loading" at the field site. APH(3')II will undergo significant degradation in the soil similar to other "free" proteins released into the environment (Stotzky 1989). Also, released APH(3')II may become adsorbed (or sequestered) onto soil particles making it unavailable. Thus, degradation and sequestering would most probably reduce the release estimates presented in this section, resulting in much lower actual exposure levels. Second, as stated above, proteins, even new and novel proteins, are constantly being added to the environment with no apparent adverse environmental effects. In addition, the FDA "Red Book" (1982) categorizes proteins into Category B, "of intermediate or unknown probable toxicity," in its level of concern classification scheme. Finally, enzymes, which include APH(3')II, generally do not raise safety concerns since very few toxic agents have enzymatic properties (Pariza and Foster 1983).

The next reason for little concern from release of APH(3')II involves a structure-activity argument. APH(3')II is an enzyme which catalyzes a specific reaction, an ATP-dependent phosphorylation of the 3'-hydroxyl group on the aminohexose moiety of a narrow spectrum of aminoglycoside antibiotics. Phosphorylation of the antibiotics interferes with the uptake and binding of these antibiotics to cellular ribosomes, thereby rendering the cells resistant to the antibiotics. This specifically defined activity functions only on antibiotic substrates and thus, would not be expected to produce any toxic effects in plants or animals. In addition, APH(3')II needs specific substrates in order to perform its catalytic activity. These substrates, ATP and aminoglycoside antibiotics, are not prevalent in the environment. Finally, populations of kanamycin-resistant bacteria exist in the soil (Henschke and

Schmidt 1989, Van Elsas and Pereira 1986). All of these bacteria do not produce APH(3')II, but certainly some of them produce it and do so constitutively (Davies 1986). This should result in a potential low-level background exposure for which no adverse effects have been observed.

The final reason for little concern from release of APH(3')II relates to history of use. The *kan*^{r} gene, and consequently APH(3')II, has been used as a "selectable marker" in numerous plants, bacteria and animals (including humans – see Section H.1.), with no apparent adverse effects.

There are two pathways by which APH(3')II may be released into the environment. The first pathway is via release of APH(3')II from crops remaining in the field after harvest. The second pathway is via wastes generated through processing or via wastes generated by humans and animals consuming the crop plants. These pathways are discussed below.

G.2.1 APH(3')II Concentration in the Environment from Agricultural Residues

As discussed in Section G.1.2.1, "Natural Transformation and Agricultural Residues," large quantities of plant material are left in the field after the harvest of a crop. In the case of crops containing the *kan*^{r} gene, this plant material remaining in the field may contain APH(3')II. This section of the EA evaluates the potential fate of this gene product by: (1) calculating the total potential yearly release of APH(3')II, and (2) calculating the yearly concentration of APH(3')II added to the soil, from transgenic tomato, cotton and rapeseed.

In any toxicological evaluation, the two critical questions are: (1) What is the potential exposure to the compound of interest, and (2) Would this level of exposure result in any adverse effects? The calculations presented in this section allow one to begin to evaluate the potential exposure of organisms in the environment to APH(3')II, but these calculations do not answer the question of what level of exposure may be toxic to organisms in the environment. The next section, "Environmental Effects of Released Substances," attempts to answer this second question, which concerns the ecotoxicity of APH(3')II.

The step-by-step calculations for each crop species are presented in Appendix EA-9 summaries of the results and the assumptions used are discussed below.

For calculating both the total potential yearly release of APH(3')II and the yearly concentration of APH(3')II added to the soil, the following assumptions were used:

- Crop plants are composed of 1% protein.
- Percentage of plant protein composed of APH(3')II is 0.1%.

Total yearly release of APH(3')II. Using the above assumptions along with the biomass of plant material per acre and the acres harvested per year yields the following estimates of release for the three crop species of interest:

- Tomato 250 metric tons per year
- Cotton 6900 metric tons per year
- Rapeseed 520 metric tons per year
- Total 7670 metric tons per year

This number can be compared to the total calculated plant biomass of 767,000,000 metric tons per year for these three crops.

Concentration of APH(3')II in soil. The calculation of the concentration of APH(3')II added to the soil is somewhat analogous to the calculation of the concentration of DNA added to the soil derived previously. The APH(3')II concentration in soil moisture was calculated assuming that all APH(3')II is extracted from the plant cells, none of the APH(3')II is degraded and all of the APH(3')II is available in the interstitial soil moisture. A further assumption implicit in this calculation is that all the APH(3')II is simultaneously extracted from the plant debris but not available to proteases for degradation to give the instantaneous concentrations listed below:

- Tomato 1.7 µg/ml
- Cotton 1.5 µg/ml
- Rapeseed 1.5 µg/ml
- Maximum concentration 1.7 µg/ml

Again, it must be remembered that these values are estimates of environmental "loading" at the field site, not estimates of actual exposure. In addition, the total yearly release of APH(3')II calculated above represent overestimates of the true values due to the fact that the fruit or seed, which is usually harvested, is included in the field biomass values used in the calculations. It should be noted that these values assume that all the

acreage of these three crops grown in the U.S. are planted with transgenic varieties.

G.2.2 APH(3')II Concentration in the Environment from Human, Animal and Processing Wastes

This pathway has already been dealt with indirectly in the previous section. In calculating the release of APH(3')II from agricultural residues, the crop biomass figures used included the weight of the entire plant. Thus, any part of the plant subsequently removed for human or animal use, or during processing has already been accounted for in our calculations of environmental release from agricultural residues. Thus, no additional APH(3')II would enter the environment through human, animal and processing wastes.

The prerequisite for this particular pathway is that active gene product, APH(3')II, reach the environment after passing through one or a combination of the following: human digestive tract, animal digestive tract, or the typical steps used in the processing of the respective product. The probability of active gene product surviving any one of these three pathways is essentially zero for the following reasons (Appendix EA-13):

- Most, if not all of the *kan*^{r} DNA ingested by humans in the form of tomatoes will be degraded in the stomach and small intestine, before it ever reaches the critical areas for potential transformation of either microorganisms (lower small intestine, cecum and colon) or post-duodenal intestinal cells (small intestine).

- Even if intact DNA is present, the probability of microorganisms capable of being naturally transformed by this exogenous DNA is very remote. The probability of epithelial cells incorporating the DNA is also extremely small and biologically irrelevant because of the short half-life of the cells.

- Most human and processing wastes, subsequent to the digestive tract or processing facility, pass through some type of sewage treatment facility. Here, additional degradation of residual gene product would occur before discharge into the environment.

- Heat steps in commercial processing and cooking will denature and inactivate the gene product APH(3')II. After short periods of time, most proteins subjected to thermal stresses exceeding 70°C become denatured (Sambrook 1989). Therefore, the heat treatment used to process foods can reasonably be expected to inactivate the enzyme. For example, commercial extraction of seed oils is facilitated by heating the seed meats (Swern 1982, Wolff 1983), which coagulates proteins in the

seed causing the oil to coalesce and flow more easily. Coagulating the proteins also decreases the affinity of the residual solid for oil, thereby making the processing more efficient (Swern 1982, Gould 1983). To enhance the effect of any heat or pressure treatment, seeds are rolled or crushed to increase their surface area. This would have the effect of liberating cytosolic enzymes that degrade proteins, further contributing to the safety of the product.

- pH is another important factor with respect to some foods. Since the pH of processed tomato products is federally regulated at pH 4.6 or less, which is far below the pH optimum of APH(3')II, no activity is expected in processed tomatoes.

- Another reason that the gene product would not be found in end products such as cottonseed or rapeseed oil is that APH(3')II is a cytosolic enzyme (Davies 1986). Such proteins maintain their tertiary structure (and therefore their function) by orienting their hydrophilic amino acids in contact with aqueous surroundings, and their hydrophobic amino acids towards the interior of the molecule. In order to preferentially separate into the lipid phase, such a protein would have to undergo major conformational changes, which would, of necessity, destroy its enzymatic activity.

Section H. Environmental Effects of Released Substances

H.1 Human Toxicity

Two clinical studies involving human gene therapy (Kasid *et al.* 1990, Blaese *et al.* 1990) are using the *kan*^{r} selectable marker (from Tn*5*) identical to that present in Calgene's genetically engineered cotton, rapeseed and tomato (Kantoff *et al.* 1988). These clinical studies represent a human *in vivo* safety evaluation of the *kan*^{r} selectable marker gene. No adverse effects of the *kan*^{r} selectable marker gene or gene product have been observed.

H.1.1 TIL Therapy

The first study involves insertion of the *kan*^{r} selectable marker gene into TIL (tumor-infiltrating lymphocyte) cells infused into cancer patients. TIL cells were first described in 1986. In rodent experiments, TIL cells (incubated with interleukin 2) were observed to be 50-100 times more potent in mediating tumor rejection than lymphokine-activated killer (LAK) cells. Early human experiments showed that TIL cells recognize the patient's own cancer cells and are not effective in another patient. TIL therapy is a form of "adaptive immunotherapy" in which a patient's own lymphocytes are removed, stimulated with interleukin 2, grown to large numbers in culture and reintroduced to the patient as immunotherapy for metastatic cancer. Of the first 25 patients treated with TIL therapy, each of whom had advanced melanoma or kidney cancer that did not respond to other treatments, half showed tumor regression of 50% or more (Potera 1989). Investigators, puzzled why the therapy worked so well in some patients but not others, sought to answer the questions: how long do the infused TIL cells persist *in vivo*; where are they located in the body; does their longevity or location correlate with clinical effects; and what are the functional characteristics of the TIL cells that are recovered from both tumor and distant sites? In the new clinical studies, the *kan*^{r} selectable marker was incorporated into the TIL cells to serve as a "tag" allowing the distribution of these cells to be monitored.

H.1.2 ADA(-)SCID Therapy

Severe combined immune deficiency (SCID) is a lethal genetic disease characterized by the virtual absence of all T and B cell immune function (Kantoff *et al.* 1988). Most affected infants die of opportunistic infections before the age of two. In one-fourth of SCID cases, a deficiency of the purine catabolic enzyme adenosine deaminase (ADA) has been identified. ADA catalyzes the conversion of adenosine (Ado) and deoxyadenosine (dAdo) to inosine and deoxyinosine. In the absence of this enzyme, dAdo accumulates intracellularly and is phosphorylated to dATP (which is normally present only at low levels in mammalian cells). The high levels

of dATP are thought to result in cellular toxicity. T cells contain the highest levels in the body of the enzymes that phosphorylate dAdo to dATP, which likely accounts for the observed selective toxicity toward the immune system. Human gene therapy for this disease involves removal of some T lymphocytes from the patient, insertion of the missing ADA gene using a vector containing the *kan*^{r} selectable marker gene, selecting for those T cells that have acquired the ADA gene, growing the cells to large numbers in culture and reintroducing them into the patient. The clinical protocol was approved by the NIH Institutional Biosafety Committee.

H.1.3 Safety Information

Anderson *et al.* (1987) in a preclinical data document submitted to the NIH Recombinant DNA Advisory Committee commented that "since eukaryotic cells are already resistant to the aminoglycosides used clinically, there appears to be no risk from expression of the *neo*^{r} (*kan*^{r}) gene" in the transformed bone marrow cells. No clinically observable adverse side-effects resulting from the presence of the *kan*^{r} selectable marker gene and gene product have been noted in the studies performed to date. Blaese and Anderson (1990) reported that they "have observed no untoward effects of expression of the *neo*^{r} gene in T cells either *in vitro* or in mice, monkeys, or humans who have received T cells expressing this enzyme." In comments presented to the NIH Institutional Biosafety Committee (March 7, 1990), Dr. W. French Anderson stated that no antibodies to the *kan*^{r} gene product had been detected in patients receiving the TIL therapy. He did indicate that sensitive assays for the *kan*^{r} gene product, not currently available, are needed. In comments before the same committee, related to both the TIL and ADA(-)SCID studies, Dr. Michael Blaese reported that "no evidence of gene product toxicity has been observed with the genes inserted to date."

H.2 Non-Human Toxicity

The previous sections of the EA have detailed the production, use, disposal and transformation potential of the *kan*^{r} gene and have concluded that there is a very low probability of the gene or its products getting much beyond the agricultural sites of interest, and that it is equally unlikely that actual "release" would result in a phenotype with a selective and/or competitive advantage in the wild.

However, for the sake of completeness, the assessment can be extended to the unlikely scenario of APH(3')II remaining in the environment following crop harvest or somehow transported away from the site as a result of *kan*^{r} gene transformation. The issue would then become the

potential for adverse environmental effects.

Toxicity tests have not been conducted, nor are they reported in the literature for APH(3')II.

In addition, APH(3')II is naturally occurring in the environment. As a naturally-occurring protein produced by soil bacteria, it is very unlikely that microbes, plants, or wildlife would be adversely impacted. Also, there is no evidence that "free" proteins or enzymes will be toxic to animals or plants and that rapid degradation of "unprotected" proteins is likely, as natural mechanisms already exist to degrade naturally-occurring APH(3')II (i.e., one does not find APH(3')II accumulating in the soil).

Finally, Calgene's proposed use of the *kan*^{r} gene would not be expected to significantly alter the concentration or distribution of the otherwise naturally-occurring gene product. There would be no new or unique routes of exposure. Even in the event that the *kan*^{r} gene was somehow released into the wild, survived, multiplied, and came into contact with a susceptible biological system, there is no basis to expect that it would cause any harm.

Section I. Use of Resources and Energy

Cotton, rapeseed and tomato varieties containing the *kan*^{r} gene will simply substitute for crops not containing the *kan*^{r} gene. Therefore, requirements of natural resources, including land use, minerals and energy, required to produce, transport, use and/or dispose of crops containing the *kan*^{r} gene do not differ from crops that do not contain this gene.

Calgene has conducted 17 field trials with cotton (at 59 different sites), 6 with rapeseed (at 20 sites) and 15 with tomato (at 20 sites) and therefore has considerable experience in growing transgenic crops. Calgene has found no differences between transgenic cotton, rapeseed or tomato with their non-transgenic counterparts in the requirements of natural resources, including land use, minerals and energy, required to produce, transport, use and/or dispose of these crops. Calgene's field trial reports (Appendix EA-16) and the Interpretative Ruling from the USDA on the FLAVR SAVR™ tomato (Appendix EA-11) support this conclusion.

No effects are anticipated upon endangered or threatened species or upon property listed in or eligible for listing in the National Register of Historic Places.

Section J. Mitigation Measures

No adverse environmental effects will result from this action. Consequently, mitigation measures are not necessary.

Section K. Alternatives to the Proposed Action

No adverse environmental effects will result from this action. Consequently, alternatives to the proposed action are not necessary for this food additive petition.

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Section M. Certification

The undersigned official certifies that the information presented is true, accurate and complete to the best of knowledge of Calgene, Inc.

June 3, 1993

Don Emlay, Director, Regulatory Affairs Date

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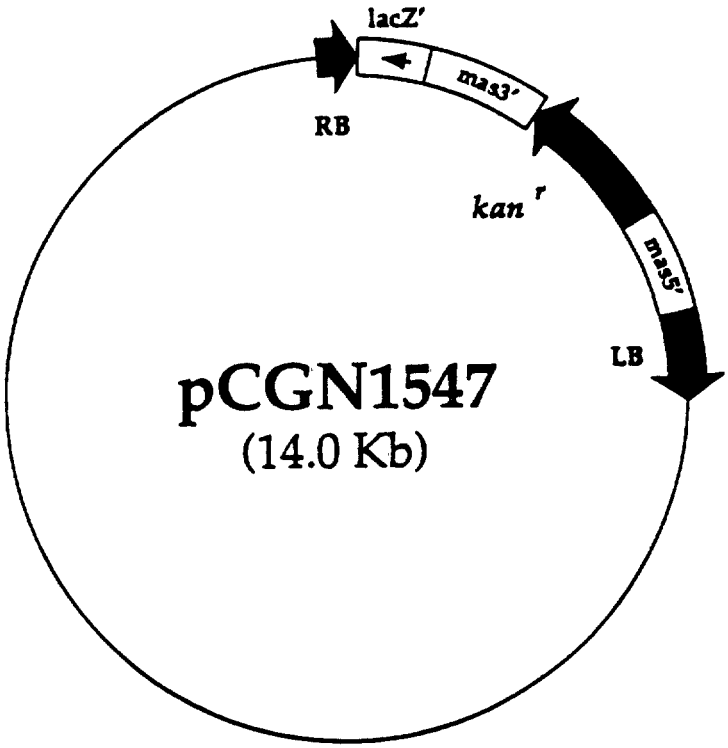
Section O. Appendices (Attached)

[redacted]

APPENDIX EA-1

Nucleotide Sequences for the *kan*^{r} Gene Constructs



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 139 GTCCCATGTGGATCACTCCGTTGCCCCGTCGCTCACCGTGTTGGGGGGAAGGTGCACATGGCTCAGTTC  207

 208 TCAATGGAAATTATCTGCCTAACCGGCTCAGTTCTGCGTAGAAACCAACATGCAAGCTCCACCGGGTGC  276

 277 AAAGCGGCAGCGGCGGCAGGATATATTCAATTGTAAATGGCTTCATGTCCGGGAAATCTACATGGATCA  345

 346 GCAATGAGTATGATGGTCAATATGGAGAAAAAGAAAGAGTAATTACCAATTTTTTTTCAATTCAAAAAT  414

 415 GTAGATGTCCGCAGCGTTATTATAAAATGAAAGTACATTTTGATAAAACGACAAATTACGATCCGTCGT  483

 484 ATTTATAGGCGAAAGCAATAAACAAATTATTCTAATTCGGAAATCTTTATTTCGACGTGTCTACATTCA  552

 553 CGTCCAAATGGGGGCTTAGATGAGAAACTTCACGATCGCCTCGAGGGGCCAACAGAGCCTGGCGTTCCC  621

 622 CTTTTGCATTGAGACCGATGTTCGTTCCGGAACCTTGCACGCCCCAGAGCTTCTCACCGTTCACGACAA  690

 691 TTTCCTTCTCGTTGAGGTCGGTCGCGCCATGTCGGATGAAATAAAAACTTTTGATACCAGCGGGGGCCT  759

 760 TCGCAGAGCCGAGGTAGGTCTGAGAAATTGGCATTTTCACGTGTGGAAGATATGAATTTTTTTGAGAAA  828

 829 CTAGATAAGATTAATGAATATCGGTGTTTTGGTTTTTTCTTGTGGCCGTCTTTGTTTATATTGAGATTT  897

 898 TTCAAATCAGTGCGCAAGACGTGACGTAAGTATCTGAGCTAGTTTTTATTTTTCTACTAATTTGGTCGT  966

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1036 TTCTTGATCCGCAGCCATTAACGACTTTTGAATAGATACGCTGACACGCCAAGCCTCGCTAGTCAAAAG 1104

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1174 TCAGAAATGGATAAATAGCCTTGCTTCCTATTATATCTTCCCAAATTACCAATACATTACACTAGCATC 1242

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1450 TGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTCCAGGACGAGGCAGCGCGGCTATCGTGGCTGGC 1518
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1795 GGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGT 1863
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2071 CTTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACG 2139
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2140 CCCAACCTGCCATCACGAGATTTCGATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTT 2208

2209 TTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCACCCCGGA 2277

2278 ATTCCAGCTTTCACGTGCTTGATACCAAGGATTTCGACACTTCGGGCATAGACCCAGAAGTCCTTAAAA 2346

2347 TCGTCGGCGCCCTCATTTGCGAAGCTCCCTGAAACCTTGGACTCCCATGTTGGCAAAGGCAACCAAACA 2415

2416 AACAATGAATGATCCGCTCCTGCATATGGGGCGGTTTGAGTATTTCAACTGCCATTTGGGCTGAATTGA 2484

2485 AGACATGCTCCTGTCAGAAATTCCGTGATCTTACTCAATATTCAGTAATCTCGGCCAATATCCTAAATG 2553

2554 TGCGTGGCTTTATCTGTCTTTGTATTGTTTCATCAATTCATGTAACGTTTGCTTTTCTTATGAATTTTC 2622

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3313 CCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGT 3381

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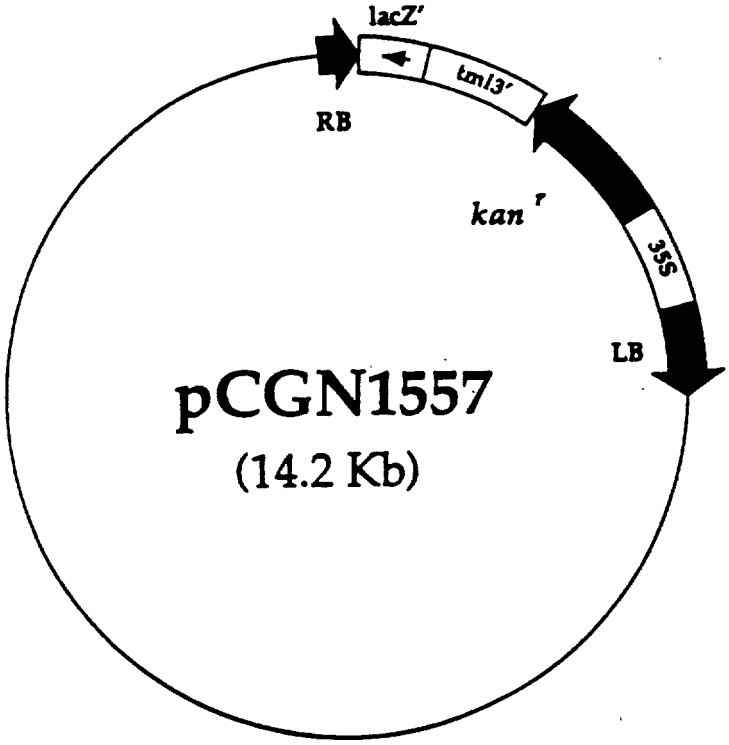
3658 TGGAGTGCAGCCCATTTCACCGGACAAGTCGGCTAGATTGATTTAGCCCTGATGAACTGCCGAGGGGAA 3726

3727 GCCATCTTGAGCGCGGAATGGGAATGGATTTCGTTGTACAACGAGACGACAGAACACCCACGGGACCGA 3795

3796 GCTTCGGAACCGCCGGTGATGATCCGGGCAGAAGATCTG 3834

Gene Sequences

Gene	Sequence	Reference	Reference Sequence
Left Border	8-587	Barker 1983	626-1205
mas 5′	599-1277	Barker 1983	20806-20128
kan^r	1296-2274	Beck 1982	1540-2518
mas 3′	2283-3053	Barker 1983	19243-18473
Lac Z′	3072-3516	Yanisch-Perron 1985	680-236
Right Border	3517-3801	Barker 1983	13992-14276
Overdrive T-Strand	3623-3646	Peralta 1986	


lacZ'
tml3'
RB
kan r
35S
LB
pCGN1557
(14.2 Kb)

PCGN1557 Linear LENGTH = 4261

```
   1 AGATCTGCGTGAACGTCGGCTCGATTGTACCTGCGTTCAAATACTTTGCGATCGTGTTGCGCGCCTGCC   69

  70 CGGTGCGTCGGCTGATCTCACGGATCGACTGCTTCTCTCGCAACGCCATCCGACGGATGATGTTTAAAA  138

 139 GTCCCATGTGGATCACTCCGTTGCCCCGTCGCTCACCGTGTTGGGGGGAAGGTGCACATGGCTCAGTTC  207

 208 TCAATGGAAATTATCTGCCTAACCGGCTCAGTTCTGCGTAGAAACCAACATGCAAGCTCCACCGGGTGC  276

 277 AAAGCGGCAGCGGCGGCAGGATATATTCAATTGTAAATGGCTTCATGTCCGGGAAATCTACATGGATCA  345

 346 GCAATGAGTATGATGGTCAATATGGAGAAAAAGAAAGAGTAATTACCAATTTTTTTTCAATTCAAAAAT  414

 415 GTAGATGTCCGCAGCGTTATTATAAAATGAAAGTACATTTTGATAAAACGACAAATTACGATCCGTCGT  483

 484 ATTTATAGGCGAAAGCAATAAACAAATTATTCTAATTCGGAAATCTTTATTTCGACGTGTCTACATTCA  552

 553 CGTCCAAATGGGGGCTTAGATGAGAAACTTCACGATCGCCTCGAGGAGCTTCACGCTGCCGCAAGCACT  621

 622 CAGGGCGCAAGGGCTGCTAAAGGAAGCGGAACACGTAGAAAGCCAGTCCGCAGAAACGGTGCTGACCCC  690

 691 GGATGAATGTCAGCTACTGGGCTATCTGGACAAGGGAAAACGCAAGCGCAAAGAGAAAGCAGGTAGCTT  759

 760 GCAGTGGGCTTACATGGCGATAGCTAGACTGGGCGGTTTTATGGACAGCAAGCGAACCGGAATTGCCAG  828

 829 CTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTTTCTTGCCGCCAAGGA  897

 898 TCTGATGGCGCAGGGGATCAAGATCCGTCCTATCTGTCACTTCATCAAAAGGACAGTAGAAAAGGAAGG  966

 967 TGGCACCTACAAATGCCATCATTGCGATAAAGGAAAGGCTATCGTTCAAGATGCCTCTGCCGACAGTGG 1035

1036 TCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAA 1104

1105 GCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGA 1173

1174 CCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACACGCTGAAATCACCAGTCTCTCTCTAC 1242

1243 AAATCTATCTCTCTCTATTTTCTCCATAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCT 1311

1312 TATAGGGTTTCGCTCAGATCCGTCGACGTCGAGGAATTCCCCGGATCGTTTCGCATGATTGAACAAGAT 1380
                                                        METIleGluGlnAsp

1381 GGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACA 1449
     GlyLeuHisAlaGlySerProAlaAlaTrpValGluArgLeuPheGlyTyrAspTrpAlaGlnGlnThr

1450 ATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACC 1518
     IleGlyCysSerAspAlaAlaValPheArgLeuSerAlaGlnGlyArgProValLeuPheValLysThr

1519 GACCTGTCCGGTGCCCTGAATGAACTCCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGC 1587
     AspLeuSerGlyAlaLeuAsnGluLeuGlnAspGluAlaAlaArgLeuSerTrpLeuAlaThrThrGly

1588 GTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTG 1656
     ValProCysAlaAlaValLeuAspValValThrGluAlaGlyArgAspTrpLeuLeuLeuGlyGluVal

1657 CCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATG 1725
     ProGlyGlnAspLeuLeuSerSerHisLeuAlaProAlaGluLysValSerIleMETAlaAspAlaMET
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[redacted] 000346

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1726 CGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGA 1794
     ArgArgLeuHisThrLeuAspProAlaThrCysProPheAspHisGlnAlaLysHisArgIleGluArg

1795 GCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCG 1863
     AlaArgThrArgMETGluAlaGlyLeuValAspGlnAspAspLeuAspGluGluHisGlnGlyLeuAla

1864 CCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGC 1932
     ProAlaGluLeuPheAlaArgLeuLysAlaArgMETProAspGlyGluAspLeuValValThrHisGly

1933 GATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTG 2001
     AspAlaCysLeuProAsnIleMETValGluAsnGlyArgPheSerGlyPheIleAspCysGlyArgLeu

2002 GGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAA 2070
     GlyValAlaAspArgTyrGlnAspIleAlaLeuAlaThrArgAspIleAlaGluGluLeuGlyGlyGlu

2071 TGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGC 2139
     TrpAlaAspArgPheLeuValLeuTyrGlyIleAlaAlaProAspSerGlnArgIleAlaPheTyrArg

2140 CTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCAACCTGC 2208
     LeuLeuAspGluPhePhe

2209 CATCACGAGATTTCGATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACG 2277

2278 CCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCACCCCGGAATTCCCGGGG 2346

2347 GAGGAATACACTAGAGGAAGGAGAAGATGACGACGATGAGATGGACGATGAAGGGGAGGCTGGTGGAGC 2415

2416 GGAACCAAGAGAGTGTCAGATCGGAAACCTTATCAATTATCCGATCATTGCTTTAGGGTCATGCGATCT 2484

2485 TTCCGCATAATTCCCGTCGCCGACACCTAATAAAGTCGGCTAATCTATGTGATTGAGTGTGTCTTGACT 2553

2554 TTGTTATTTTGCATGTTTCCAATGTCATTTAGTAACGAAATAAACGTTATCCTCTTCTAAAAGCAGGCT 2622

2623 GTGTTTTCGGCAAACATCGCCACCCATCGCTAGTTTTTCTAAAAGTGTTCTAAGCTAGCCTGGTAATAA 2691

2692 TCTATACGAGCTTATATTTCTAATCATTGCCGAAAAATCCTGTTTCGAAATAATTTTGTAACTCTCTTT 2760

2761 AATATCACCACGATCACACAAGAAGAAGAATTAAATATAACATTTATCAAGCCCACGATGAACATGGCG 2829

2830 AAAATTACAAGCGAACACAATTGTCTTATTCATTAATAATTAATTCAACAAAGTTCTCAGACACTCCTT 2898

2899 GATTTAACAGAAGATTTTATAAGCACCTCTATGATGCAACAACCAAAACGACATAGACTTCGAAATCCC 2967

2968 GCCCCATAAAAACTAGATAGCACCATCTAACTCCACGTCTTCAAGAGCAGCCTCCGGGAGGAAAGCTGA 3036

3037 ACAGCCCACAGCGATCAAAAGATAATCCAATGGTTGGCCGGCGCTTGGCATTTTCCTTTTCCAAACGTC 3105

3106 CTTGCTTTGTCCAAAAGTGTTCCGGACGACTCCTATGCGTTCGAACTCGTTGCTGGCCTGAATTGCTAG 3174

3175 AAAGTACGAGGATGAATATTCATTTTTGCTGTAGTTGATCCGACCACCGTCTCCGCTTAAGGCTTGCAG 3243

3244 TTGCGCCCTCACCTGCCGGAATGTATAACCATCAGAATAGGGTTCCAGTGTCGTGGCAAGCAATCCATC 3312

3313 TGAAGCATTTGAAACTAGAACCATTCTTTGCCGACATAACCGATAATTCTCTTTATCGAGATAAATGTA 3381

3382 CAGAAATTCCCCGCCAGGATTGATGATGTCGCCAGACTTAGTGTGTAGATCGGTTTGAAACTTTTCCCG 3450
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3451 ATAAACTTTTGAAAGCTCCGAGAGTTCACCCCCTCGAGAGCTTGGCGCCCAATACGCAAACCGCCTCTC 3519

3520 CCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAG 3588

3589 CGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCT 3657

3658 CGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGAA 3726

3727 TTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCACTGGCCGTCGT 3795

3796 TTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTT 3864

3865 CGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGG 3933

3934 CGAATGGCGCAGACCTCAATTGCGAGCTTTCTAATTTCAAACTATTCGGGCCTAACTTTTGGTGTGATG 4002

4003 ATGCTGACTGGCAGGATATATACCGTTGTAATTTGAGCTCGTGTGAATAAGTCGCTGTGTATGTTTGTT 4071

4072 TGATTGTTTCTGTTGGAGTGCAGCCCATTTCACCGGACAAGTCGGCTAGATTGATTTAGCCCTGATGAA 4140

4141 CTGCCGAGGGGAAGCCATCTTGAGCGCGGAATGGGAATGGATTTCGTTGTACAACGAGACGACAGAACA 4209

4210 CCCACGGGACCGAGCTTCGGAACCGCCGGTGATGATCCGGGCAGAAGATCTG 4261

Gene Sequences

Gene	Sequence	Reference	Reference Sequence
Left Border	8-587	Barker 1983	626-1205
Tn5	599-922	Auerswald 1981	1195-1518
CaMV 35S	927-1327	Gardner 1981	7146-7546
kan^r	1355-2333	Beck 1982	1540-2518
tml 3′	2344-3481	Barker 1983	11207-10070
Lac Z′	3499-3943	Yanisch-Perron 1985	680-236
Right Border	3944-4228	Barker 1983	13992-14276
Overdrive T-Strand	4050-4073	Peralta 1986	

APPENDIX EA-2

Analytical Methodology and Typical Results

[redacted]

D.1 Relevant Analyses

Several analytical procedures are described below for quantitating the levels of the *kan*^{r} gene and its translation product, the enzyme APH(3')II, in transgenic plants, as well as for estimating the degradation of the gene and gene product under simulated gastrointestinal tract conditions. The levels of both the *kan*^{r} gene and the APH(3')II enzyme might be expected to vary somewhat among different plants (e.g., tomato, cotton, and rapeseed), while the levels of the APH(3')II enzyme can vary among different tissues of the same plant (e.g., leaf vs. fruit). The data provided below represent typical levels observed to date in transgenic tomatoes.

D.1.1 Quantitation of *kan*^{r} Gene

Quantitation of the number of *kan*^{r} genes that have been integrated into the genome of a genetically engineered crop plant is conducted on individual transgenic plants. Specific information will therefore be gathered on a product by product basis. The Southern analysis described in this section serves as an example of how that information will be gathered.

D.1.2 Quantitation of DNA Degradation Under Simulated Gastrointestinal Tract Conditions

In order to determine whether DNA would survive intact (i.e., in large enough fragments to encode the *kan*^{r} gene) during passage through the gastrointestinal tract, bacteriophage λ DNA (serving as a model DNA) was subjected to simulated gastric and intestinal conditions for various time intervals and then analyzed for size distribution.

D.1.3 Quantitation of APH(3')II Gene Product

The amount of APH(3')II present in a samples of ripe fruit from transgenic tomato plants has been determined. In addition, the extent of degradation of APH(3')II under simulated gastrointestinal tract conditions was evaluated.

D.2 Methods Needed to Detect and Determine Concentration

D.2.1 *kan*^{r} Gene

The *kan*^{r} selectable marker gene may be quantitated by Southern Blot Analysis. This procedure involves electrophoretic separation of DNA (extracted from plant tissue) in an agarose gel, transfer of the DNA onto a solid support, and detection with a nucleic acid probe that is specific for a particular sequence of nucleotides. The protocol for this procedure was described by Southern (1975).

D.2.2 Degradation of Model DNA

The size distribution of bacteriophage λ exposed to simulated gastrointestinal tract conditions is also assessed by Southern Blot analysis.

D.2.3 APH(3')II Gene Product

The amount of APH(3')II protein may be quantitated using both immunological and enzymatic assays. Immunological methods detect APH(3')II protein, whether or not it is enzymatically active; whereas, enzymatic assays monitor only active enzyme.

D.2.3.1 Western Blot Analysis

Analysis of APH(3')II levels by Western Blot involves electrophoretic separation of proteins on a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE, Laemmli 1970), transfer to a solid support (nitrocellulose), and probing with an antibody specific for the target protein (rabbit APH(3')II antiserum). The APH(3')II protein is visualized using secondary mouse anti-rabbit antibodies coupled to alkaline phosphatase and detected using the appropriate color reagents. The Western Blot procedure is described by Towbin et al. (1979).

D.2.3.2 APH(3')II Enzymatic Assay

Levels of enzymatically active APH(3')II can be measured by an assay in which the phosphorylation of neomycin or kanamycin by ^{32}P-labelled ATP is monitored. This procedure has been described by Radke et al. (1988).

D.2.3.3 Kanamycin Germination Assay

The presence of the *kan*^{r} gene in genetically engineered plants can be identified by a germination assay in which seeds are sprouted in media containing the antibiotic kanamycin. In general, *kan*^{r} gene-containing (resistant) seedlings will exhibit long branching roots, while non-*kan*^{r} gene containing (susceptible) seedlings will have very short unbranched roots with purple stems. A protocol for this procedure is described in Appendix D-1.

D.3 Sample Preparation and Analysis

D.3.1 *kan*^{r} Gene Samples

Extracts of DNA from tomato, rapeseed, and cotton transgenic plants are prepared according to the protocols included in Appendix D-2

Tomato genomic DNA isolated from a non-transformed control *L. esculentum* cv. UC82B plant and from an individual UC82B plant that had been transformed with Calgene construct pCGN1557 (see Appendix A-5) was digested to completion with restriction endonucleases BamHI and EcoRI (Boehringer Mannheim, Mannheim, Germany). The resulting DNA fragments were precipitated by adding one-tenth volume 3M sodium acetate and 2 volumes of ethanol and incubating at -80°C for approximately 2 hours. After centrifugation for 10 minutes at 12,000xg pellets were resuspended in 10 µl glass distilled water and 2 µl of gel-loading buffer (0.25% bromophenol blue, 15% Ficoll (type 400) in H_2O) was added to each sample. The samples were then loaded onto a 0.7% agarose gel. The gel was subjected to electrophoresis at 75V for approximately 5 hours, photographed, and prepared for transfer to a nylon membrane (0.45 µm, MSI, Westboro, MA). Preparations for transfer consisted of gentle shaking in Southern denaturing solution (Maniatis 1982) twice for 15 minutes each time and in Southern neutralizing solution (Maniatis 1982) for 30 minutes. The DNA in the gel was then transferred to the membrane overnight by capillary action using 20X SSC (Maniatis 1982).

The membrane was then baked for 2 hrs at 80°C. The membrane was prehybridized for >6 hrs. at 42°C in 10 mls of prehybridizing solution

(described below for DNA degradation Southern). The membrane was then hybridized in the same solution to which had been added a denatured EcoRI fragment encompassing the *kan*^{r} gene coding region and that had been radiolabelled by the nick translation method (Maniatis 1982) using a labelling kit provided by Boehringer Mannheim (Mannheim, Germany) and following the manufacturer's instructions. The membrane was then hybridized, again at 42°C, overnight.

Non-specific hybridization was eliminated by washing the membrane in 0.1X SSC/0.1% SDS at 55°C for 1 hour. The membrane was then wrapped in plastic wrap and used to expose Kodak XAR film using an intensifying screen at -70°C. The exposure time for the results depicted in Figure A-1 (Appendix A-4) was approximately 16 hours.

D.3.2 DNA Degradation Samples

Each original sample consisted of 3 μg of high molecular weight DNA (λ phage DNA, New England Biolabs, Beverly, MA) in 3 ml simulated gastric fluid, pH 1.9. The simulated gastric solution is that specified in the 1990 edition of the United States Pharmacopeia, The National Formulatory (USP/NF 1990), and is prepared by dissolving 2.0 g of sodium chloride and 3.2 g of pepsin in 7.0 ml of hydrochloric acid and sufficient water to make 1000 ml. The samples were incubated at 37°C for various periods of time and then brought to pH 6.0 by the addition of 220 μl of 1N sodium hydroxide. One-tenth volume 3M sodium acetate was added and the DNA was then precipitated by adding 2 volumes ethanol and cooling to -20°C overnight.

After centrifugation for 30 min at 30,000 rpm in a Beckman SW50.1 rotor, pellets were resuspended in a minimal volume of water and then brought to a total volume of 2.4 ml with simulated intestinal fluid. The simulated intestinal solution is that specified in the 1990 edition of the United States Pharmacopeia, The National Formulatory (USP/NF 1990). A simulated human intestinal fluid test solution is prepared by dissolving 6.8 g of monobasic potassium phosphate in 250 ml of water, mixing, and adding 190 ml of 0.2 *N* sodium hydroxide and 400 ml of water. Next 10.0 g of pancreatin is added. After mixing, the resulting solution is adjusted to pH 7.5 ± 0.1 by the addition of 0.2 *N* sodium hydroxide. Sufficient water is then added to make 1000 ml. Tap water rather than purified water was used to prepare the solution. (Magnesium ions are present in human extracellular fluids although much less is known about the concentration of this ion and the regulation of its concentration in body fluids than is known about potassium and sodium concentrations [Guyton 1986]. Manganese, a trace element, is known to be a part of the body enzyme system [Fleck 1976]. Because the simulated intestinal fluid formula used for this experiment was designed for use in determining the degradation times for DNA, it does not call for addition of magnesium or manganese even though these elements are present in extracellular fluids. The DNase I derived from the pancreas requires, however, Mn^{+2} to hydrolyze double-stranded DNA and Mg^{+2} to hydrolyze single-stranded DNA (Maniatis 1982). The simulated intestinal fluid used for this experiment, therefore, was prepared using tap water in an attempt to add trace amounts of manganese and magnesium ions to this solution.) The samples were incubated at 37°C for various periods of time at which point 400μl aliquots were withdrawn from the incubating solution, placed in a microfuge tube containing 40 μl 0.25 M EDTA, mixed well and kept on ice.

Once all time points had been collected, the samples were extracted with phenol, to eliminate the gastric and intestinal fluid proteins, and the aqueous phases extracted with chloroform. A few micrograms of tRNA were added to each final aqueous sample to aid in the precipitation of small amounts of DNA. The DNA was then precipitated by adding 50 µl of 3M sodium acetate and 2 volumes (1ml) of ethanol to each sample and cooling to -70°C overnight.

After centrifugation for 10 min at 12,000xg, pellets were resuspended in 15 µl glass distilled water and 3 µl of gel-loading buffer (0.25% bromophenol blue, 15% Ficoll type 400 in H_2O) was added to each sample. Because samples still appeared to contain some protein they were heated to 65°C for 5 min and subjected to centrifugation at <12,000xg for 1 min prior to being loaded onto a 2% agarose gel. The gel was subjected to electrophoresis for 3 hrs at 100 V, photographed, and prepared for transfer to a nylon membrane (0.45µm, MSI, Westboro, MA). Preparations for transfer consisted of gentle shaking in 0.25 N HCl for 15 min, Southern denaturing solution (Maniatis 1982) twice for 15 min each time, and Southern neutralizing solution (Maniatis 1982) for 30 min. The DNA in the gel was then transferred to the membrane overnight by capillary action using 20X SSC (Maniatis 1982).

The membrane was then baked for 2 hrs at 80°C and then prehybridized for 6 hrs at 42°C in 10 mls of a solution containing: 5 mls formamide, 2.5 mls 20X SSC, 0.5ml 1M sodium phosphate buffer, 0.5ml 100X Denhardt's solution (see Maniatis 1982), 0.1ml 10% SDS, 0.2 ml 0.25 M EDTA, and 0.1ml 10mg/ml denatured salmon sperm DNA. The membrane was then hybridized in the same solution to which had been added a denatured radiolabelled λ phage DNA probe that had been prepared by the random priming method using a labelling kit provided by Boehringer Mannheim (Mannheim, Germany) and following the manufacturer's instructions. The membrane was hybridized, again at 42°C, overnight.

Non-specific hybridization was eliminated by washing the membrane in 0.1X SSC/0.1% SDS at 55°C for 1 hr. The membrane was then wrapped in plastic wrap and used to expose Kodak XAR film using an intensifying screen at -70°C. The exposure time for the results depicted in Figure D-1 was 15 hrs.

D.3.3 APH(3')II Gene Product Samples

For Western blot analysis, one gram of ripe fruit tissue was ground to a powder in liquid N_2 and added to 2 ml of boiling sample buffer (0.125M Tris, pH 6.8, 2% SDS, 10% glycerol, and 0.05% bromphenol blue). β-Mercaptoethanol was added to a concentration of 5%, and the samples were run on an 11.25% polyacrylamide gel with purified APH(3')II in sample buffer. Proteins were blotted to nitrocellulose and visualized by reaction with APH(3')II antiserum, mouse anti-rabbit IgG antibodies coupled to alkaline phosphatase, and color reagents as described by the vendor (Promega).

D.4 Analytical Results

D.4.1 Quantitation of *kan*^{r} Gene

As described above, 10 copies per cell represents the upper limit level of the *kan*^{r} gene in transgenic plant tissue of commercial products.

[redacted]

The results from a Southern blot analysis of genomic DNA isolated from an individual UC82B variety tomato plant that had been transformed with the *kan*^{r} gene-containing Calgene construct pCGN1557 and from a non-transformed UC82B variety tomato plant is shown in Figure A-1 (Appendix A-4). Based on the known location for sites at which the restriction endonucleases used in this experiment, BamHI and EcoRI, cut the pCGN1557 DNA (see Appendix A-1), in the transformed plant a DNA fragment of 1 kb in length and containing the *kan*^{r} gene was expected to hybridize to the DNA fragment used as a hybridization probe in this experiment. As can be seen in Figure A-1 (Appendix A-4), hybridization of a 1 kb fragment of DNA is observed in the lane containing DNA isolated from the transformed plant. No hybridization is observed in the lane containing DNA isolated from the non-transformed, control plant.

Determining the number of *kan*^{r} genes that have been inserted into an individual transformed plant can be accomplished in similar experiments through appropriate selection of both restriction endonucleases to cut the plant DNA and hybridization probes. Comparing the intensity of a hybridization signal produced using a known amount (representing a known number of copies) of pCGN1557 plasmid DNA, for example, can also be used to determine the number of *kan*^{r} genes inserted into an individual transformant.

Note on Sensitivity of the Southern Technique

10 μg/gel lane of total mammalian DNA (haploid genome = 3 x 10^6 kb) is required in order to detect gene sequences that occur at the single copy per haploid genome level (Maniatis 1982). Assuming a gene size of 1 kb, approximately 10^{-5} μg [10 μg x (1 kb gene/3 x 10^6 kb)] of DNA can be detected using the Southern technique. This level of sensitivity will be affected by the specific activity of the hybridization probe used, i.e., the amount of radioactive label incorporated per probe fragment length. Sensitivity will also be affected by the complexity of the probe DNA as compared to that of the DNA being analyzed. For example, while the specific activities (incorporated label/μg DNA) are similar for the probes in the two Southern experiments described in Section D, the probe in the *kan*^{r} gene Southern experiments only recognizes the *kan*^{r} gene sequences and will therefore all be concentrated in the 1 kb region of the blot. In the DNA degradation Southern experiment, on the other hand, there will be less total radioactivity in the probe that will recognize any particular region of the λ DNA genome because the entire 50 kb genome is represented in the 0.5 μg DNA that was labelled (as opposed to the 1 kb *kan*^{r} sequences represented in the 0.5 μg DNA labelled in the *kan*^{r} gene experiments). This difference in total radioactivity per region of DNA (approximately 1 kb/50 kb), as well as the fact that λ sequences recognized by the probe will be randomly distributed throughout the entire molecular weight range of fragments and not concentrated in a small region of the blot as in the *kan*^{r} gene experiment, reduces the sensitivity of the Southern technique in the λ DNA degradation experiment.

D.4.2 Quantitation of DNA Degradation Under Simulated Gastrointestinal Conditions

The results from a Southern blot analysis of bacteriophage λ DNA incubated with simulated gastric fluid and simulated intestinal fluid are shown in Figure D-1.

After 10 min in simulated gastric fluid and 10 min in simulated intestinal fluid (prepared using tap water), high molecular weight DNA is extensively degraded; degraded to the point that essentially no DNA of a molecular weight greater than 1kb can be detected using the hybridization technique described above (see Figure D-1).

These results may still be an underestimate of the timing or extent of DNA degradation during digestion for the following reasons:

- DNase (and RNase) activity is also present in salivary gland secretions (Zbarsky 1971) and would be starting to break down the DNA before it reaches the stomach;
- Polynucleotides in the small intestine will be further hydrolyzed by phosphodiesterases in the border membrane of intestinal cells (Berkowitz 1990); and
- Normally 3 to 5 hrs are required for passage of chyme (stomach contents) from the pylorus (marking the border between the stomach and the small intestine) to the ileocecal valve (marking the border between the small intestine and the large intestine) (Guyton 1986). Chyme is sometimes blocked for another several hours at the ileocecal valve until the person eats another meal (Guyton 1986). Thus, ingested DNA is generally bathed in pancreatic secretions for a period of time substantially longer than 10 min.

D.4.3 Quantitation of APH(3')II Gene Product

D.4.3.1 Level of APH(3')II Protein in Food Product

Western analysis of ripe tomato fruit extracts from a *mas-kan*^{r} (1436-25) and a *kan*^{r} (1264-1) transformant are shown in Figure D-2. A signal from 1436-25 (lane B) equivalent to approximately 10 ng of pure APH(3')II (lane E) was observed. This is equivalent to 175 ng of APH(3')II per gram of fresh fruit tissue. Assuming that 7% of the fruits are solids, and 7% of the solids are protein (Davies and Hobson 1981), 1 gram of fruit tissue contains 4.9 mg protein and APH(3')II represents approximately 0.004% of the total protein. Fruit with the 35S construct (lane A) produced a signal demonstrating significantly less than 10 ng of APH(3')II activity, and therefore less than 0.004% of the total protein. Additional fruit samples were analyzed in a similar manner, with the results shown in Table D-1. The estimated number of functional *kan*^{r} genes, as determined by the segregation ratio of kanamycin-resistance in germination assays, is indicated for each transformant.

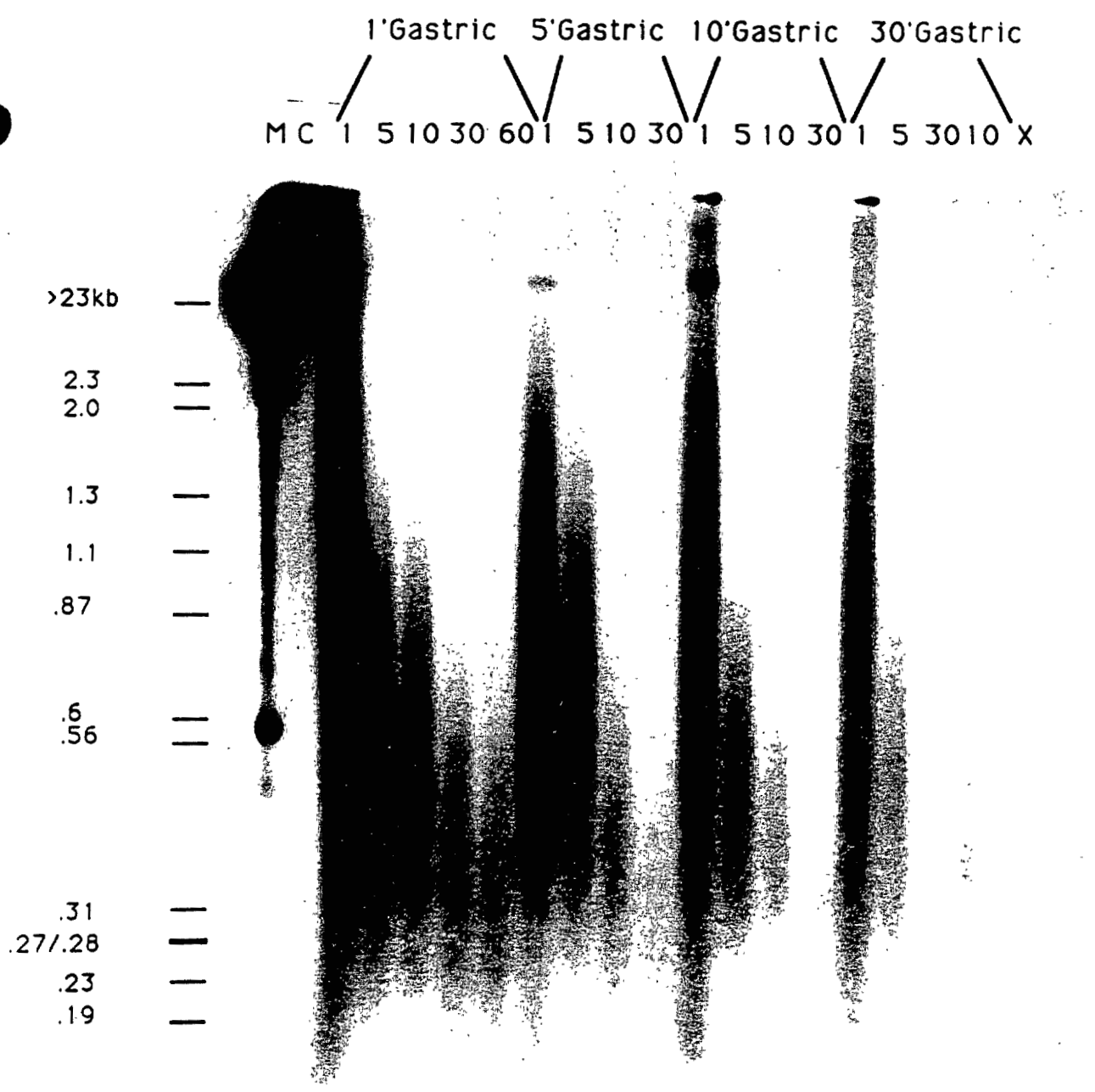

FIGURE D-1. Southern Analysis of DNA Degradation in Gastrointestinal Fluids. Bacteriophage λ DNA samples were treated as described for the indicated periods of time in simulated gastric fluid followed by an additional period of time (min., indicated by the numbers heading each column) in simulated intestinal fluid. M = molecular weight marker DNA; the molecular weight of marker DNA fragments are indicated along the left margin. 0.5 μg of undigested λ DNA was loaded into the gel lane labelled "C".

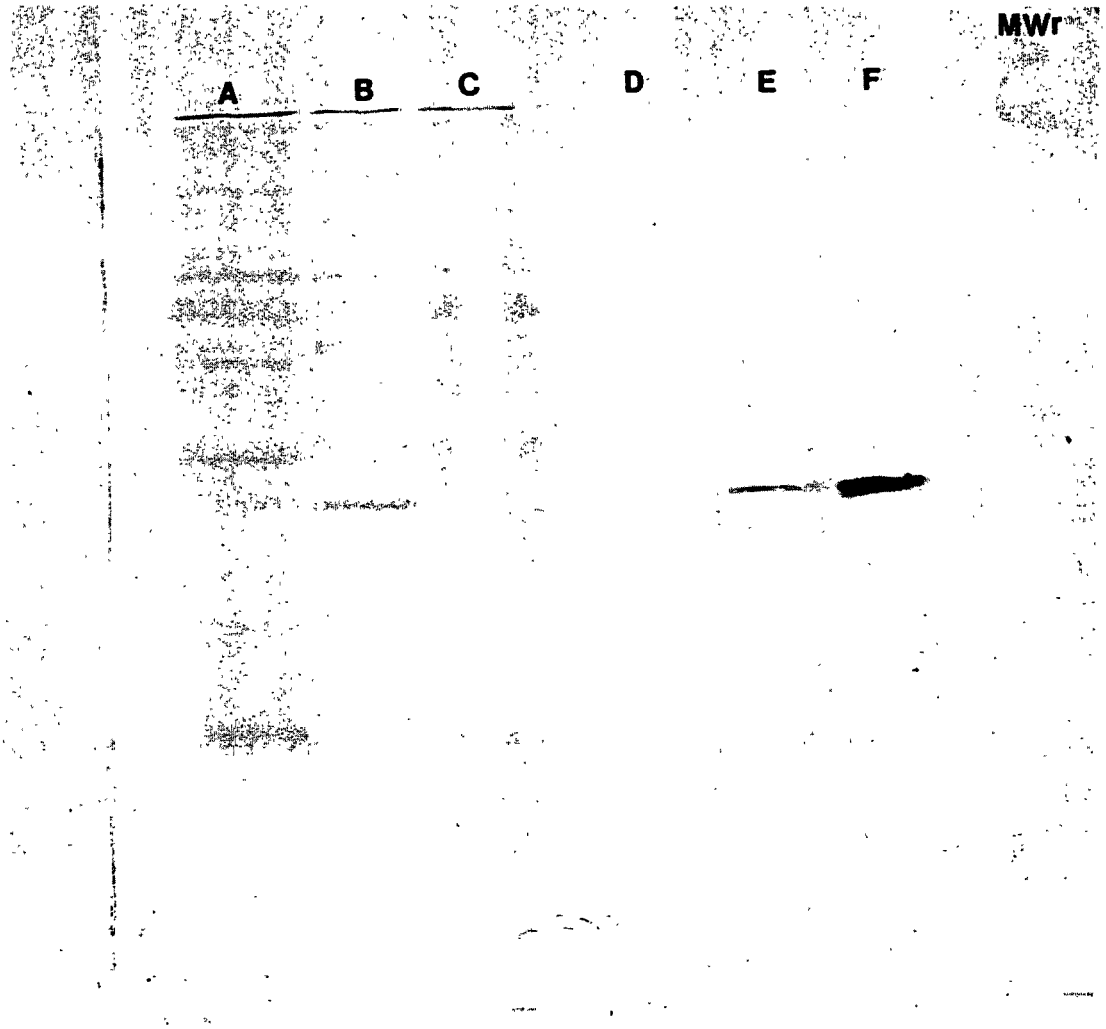

FIGURE D-2. Western Blot Analysis of APH(3')II Levels in Ripe Tomato Fruit. Samples of ripe fruit tissue were from transformants 1264-1 (35S-*kan*^{r}, lane A) and 1436-25 (*mas-kan*^{r}, lane B), as well as from an non-transformed control tomato (UC82B, lane C). Purified APH(3')II obtained from 5'Prime - 3'Prime, Inc. was added to lane D (1 ng), lane E (10 ng), and lane F (50 ng).

TABLE D-1

Analysis of Ripe Fruit Samples for APH(3')II Levels

Transformant	Segregation Ratio	Estimated Functional *kan*^{r} genes	ng APH(3')II per gm fr wt	% Total Protein
35S-*kan*				
2905-10	3:1	1	175	0.004
2905-8	15:1	2	35	0.0007
2905-2	3:1	1	140	0.003
2905-18	15:1	2	350	0.007
mas-*kan*				
7B-66	3:1	1	175	0.004
7B-107	3:1	1	175	0.004
28B-403	3:1	1	175	0.004
28B-404	3:1	1	175	0.004
7B-132	3:1	1	175	0.004

D.4.3.2 Survival of APH(3')II Protein in G.I. Tract

Purified APH(3')II was rapidly inactivated by incubation under simulated gastric conditions (0.32% pepsin, 0.2% NaCl, pH 1.2). After 20 minutes at 37°C, essentially no enzymatic activity (<1%) remained (Figure D-3).

Similarly, purified APH(3')II incubated with simulated intestinal fluid rapidly lost activity within 5 minutes at 37°C (Figure D-4). Intestinal fluid itself did not inhibit the assay.

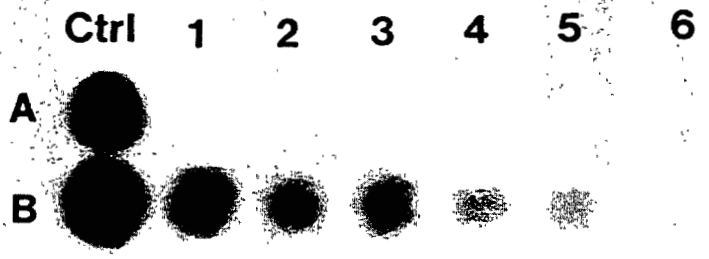

FIGURE D-3. Inactivation of APH(3')II by Pepsin at pH 1.2. Simulated gastric fluid either with 0.32% pepsin (A) or without pepsin (B) was made to 100 ng/ml with purified APH(3')II and incubated for 0 min (1), 5 min (2), 10 min (3), 20 min (4), 30 min (5), or 60 min (6) at 37°C. Aliquots were removed at the indicated times, neutralized, and measured for APH(3')II activity by the standard assay. (C) control consisting of 100 ng APH(3')II in H_2O. Results were quantitated as shown below.

Time (min)	+ pepsin		- pepsin	
	cpm	% act	cpm	% act
control[1]	3242	100	3242	100
0	118	3.6	758	23
5	114	3.5	368	11
10	110	3.4	430	13
20	24	0.7	204	6.2
30	8	0.2	154	4.8
60	20	0.6	0	0

[1] Average of two control values.

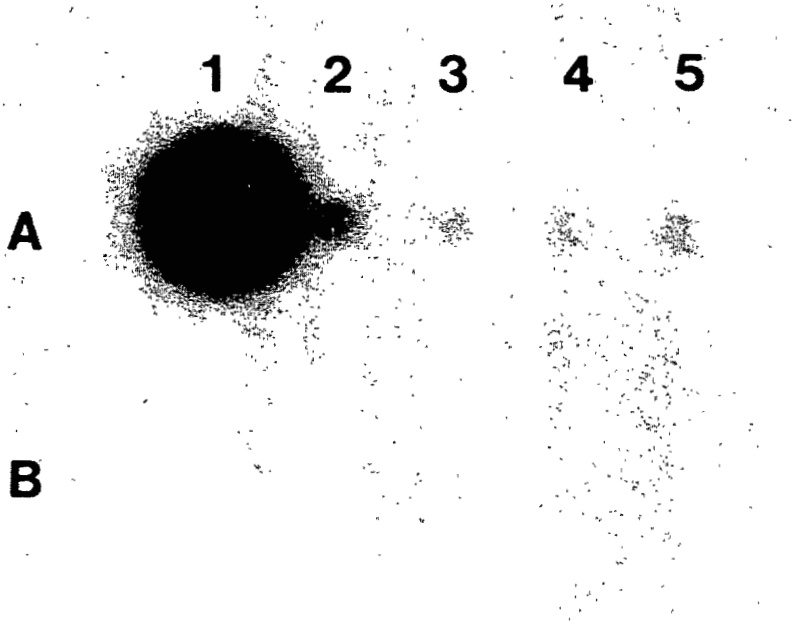

FIGURE D-4. Inactivation of APH(3')II by Simulated Intestinal Fluid. Intestinal fluid was prepared and made to 100 ng/ml with purified APH(3')II prior to incubation at 37°C for 0 min (row 1), 5 min (row 2), 15 min (row 3), 30 min (row 4), and 60 min (row 5). Aliquots were removed at those times and incubated in the standard assay with kanamycin (lane A) or no substrate (lane B). Results were quantitated as shown below.

Time (min)	cpm	% Control
0	61934	100
5	204	0.3
15	92	0.15
30	86	0.14
60	66	0.11

D.5 Discussion of Methods and Analytical Results

D.5.1 Quantitation of *kan*^{r} Gene

The upper limit levels of *kan*^{r} gene present in ripe tomatoes has been set at 10 copies per cell. The exact copy number, which will vary for different plant lines, can be determined on a product specific basis using the techniques exemplified in Figure A-1 (Appendix A-4).

D.5.2 DNA Degradation Under Simulated Gastrointestinal Tract Conditions

After 10 minutes in simulated gastric fluid and 10 minutes in simulated intestinal fluid, high molecular weight DNA is extensively degraded (Figure D-1) to the point that essentially no DNA of length greater than 1 kb (the size of the *kan*^{r} gene) can be detected using the Southern hybridization technique described above.

D.5.3 Quantitation of APH(3')II Gene Product

Based on Western blot analysis, the level of APH(3')II in a sampling of ripe tomato fruit ranges from 0.0007% to 0.007% of the total protein. Since the concentrations of APH(3')II in a given genetically engineered plant will vary, it is conservatively estimated that the upper bound limit of APH(3')II is 0.1% of the total protein in transformed plant tissue.

The experimental data also indicate that APH(3')II is essentially completely degraded under simulated gastric and intestinal conditions.

[redacted]

Appendix A-4

Analytical Results: Identity of *kan*^{r} Gene and APH(3')II Gene Product

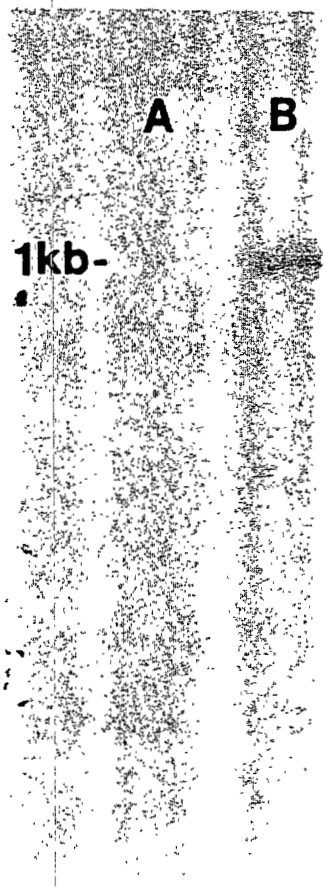

FIGURE A-1. Southern Analysis of *kan*^{r} Gene in Transformed Tomato. Tomato DNA samples were treated as described in Section D.3.1. Lane A, DNA isolated from *L. esculentum* cv. UC82B non-transformed control (approximately 30 μg); Lane B, DNA isolated from a UC82B plant transformed with pCGN1557 (approximately 5 μg). The position to which 1 kb fragments migrated on the agarose gel is indicated.

[redacted]

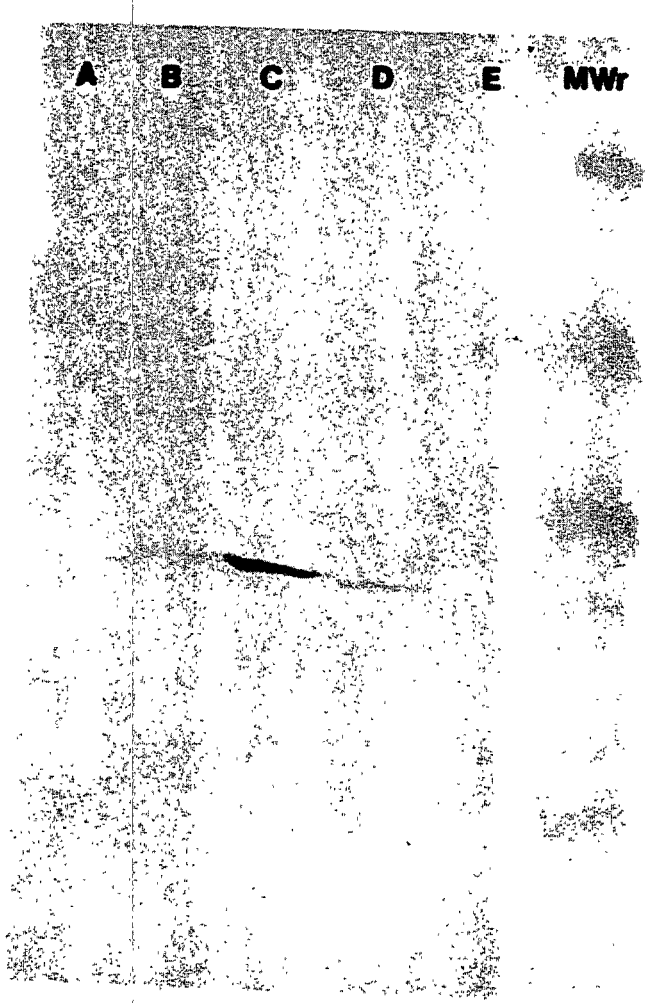

FIGURE A-2. **Identification of APH(3')II in Fruit From Tomato Plants Transformed With the *kan*^{r} Gene.** Ripe fruit extracts were prepared for Western analysis as described in Section D.3.4. Lane A, control tomato fruit; Lane B, pCGN 1557 transformed fruit (2905-10, *kan*^{r}) Lane C, 50 ng pure APH(3')II; Lane D, 10 ng pure APH(3')II; Lane E, 1 ng pure APH(3')II. Molecular weight standards are (from top to bottom of gel) 75, 50, 39, 27, and 17Kd.

[redacted]

APPENDIX D-1
Kanamycin Germination Assay

Solutions and Materials Needed

1X MS Medium Stock

4.6 g (1 packet) Murashige Minimal Organics Medium Without Sucrose (Catalog Number 510-3118) (pH adjusted) to 1 liter of double distilled H_2O.

1/10 MS 75 mg/liter Kanamycin germination medium

100 ml 1X MS stock above
900 ml double distilled H_2O
75 mg kanamycin sulfate (Boerhinger Mannheim Gmbh 106801)

1/10 MS 0 mg/liter Kanamycin germination medium

(same as above w/o the kanamycin sulfate)

Bleach sterilization solution (500 mls)

50% commercial bleach/double distilled H_20 and 2 drops Ivory dish soap as a surfactant

Magenta boxes with 3 mm paper lining bottom of box, sterilized by autoclaving

Tea infusers (spoons)

Procedure

1. Add 7 mls of 1/10 MS 75 kan medium per magenta box.
2. Sterilize seed to be tested for 5 min. in 50% bleach solution.
3. Rinse 4 times in beakers of sterile double distilled H_20.
4. Place sterilized seeds into magenta box.
5. For controls, plate untransformed seed onto 75 mg/L Kan medium and 0 mg/L Kan medium.
6. Place boxes into growth chamber for 1 week (25°C, 12 hr light/12 hr dark at 70-90 $uEm^{-2}S^{-1}$)

Scoring Seedlings

Seedlings are scored by appearance with respect to the controls. As a general guideline, susceptible seedlings have very short unbranched roots with purple stems. Resistant seedlings should have long branching roots.

Appendix D-2

DNA Isolations

Cotton DNA Isolation

Ref: Dellaporta et al "Maize DNA miniprep" (p. 36-37) in Mol. Bio of Plants: A Lab Course Manual. Cold Spring Harbor.

Ref. Cotton DNA Isolation protocol from Galau, G.A. et al (1988) MGG 211: 305-314.

Extraction Buffer:	For 100 mls
200 mM Ches/NaOH pH 9.1	20 mls 1M
200 mM NaCl	4 mls 5M
100 mM EDTA/NaOH pH 9.0	40 mls 0.25 M
2% SDS	20 mls 10%
0.5% Na deoxycholate	0.5 g
2% Nonidet NP-40	2 mls
20 mM βME	156 µl -- add fresh each time
DD H20	make to 100 mls

May store the buffer in the refrigerator for several weeks, but add fresh βME each time.

1. Lyophilize about 6 g frozen young leaf tissue

2. Pulverize in sample mill

3. Weigh out 0.5 g powder each. Place in new Oak Ridge tube. Add 0.06 g PVP, mix

4. Add 20 mls extraction buffer to each. Mix gently until all plant tissue is suspended (scrape plant material from the side of tube with clean plastic spoon)

5. Without delay, incubate in 65° water bath for 10 minutes, Mix gently every 3 minutes to ensure homogenization

6. Add 6.7 mls 5 M KAc pH 6.5 to each. Mix gently (a white precipitate should form)

7. Incubate on ice 30 minutes, mixing gently every 5 minutes. Sample should be thick with the whitish precipitate

8. Spin 20 minutes 13K in JA-17

9. Filter supernatant through miracloth into 13.3 mls room temperature (RT) isopropanol in Oak Ridge tube. Mix gently

10. Let stand at RT 10 minutes-1 hour, until DNA precipitates (if no precipitate, chill)

11. Spin down 10K JA-17 15-20 minutes or preferably spool. Air dry pellet

12. Resuspend each pellet in 4 mls TE on ice (about 1 hour) or at 4°C overnight (O/N).

13. Add 3.88 g CsCl to each sample; mix gently until dissolved (0.97 g/ml final concentration)

14. Add 300 µl 10 mg/ml EtBr to VTi 80 quick seal tube.

15. Transfer sample to tube. Top up with CsCl/TE 1 g/ml

16. Seal tube; spin in VTi 80 at 65K (or 55K), 15°C for 6 hours to overnight

17. Decelerate with no brake, pull band with pasteur pipette into falcon tube

18. Extract EtBr with 2 volumes salt saturated butanol 2 times, then 1 volume 2 times or until colorless.

19. Transfer to new falcon tube, dilute with 0.5 ml of H_2O

20. Precipitate for at least 1 hour with 1/10 vol 5 M NH_4Ac and 1 vol RT isopropanol or 2 vol EtOH. Chill only slightly if necessary, or CsCl will precipitate. Note: If an interface forms or sample becomes cloudy, you must dilute with more H_2O and alcohol.

21. Spin down at 10K in JA-17 or 11K JA-20.1 for 20 minutes (or more).

22. Air dry pellet, resuspend in 25-50 µl TE (minimum of 1 hour to O/N)

23. Dilute 2 µl in 0.6 ml H_2O; scan at 300-200 nm

24. Digest 10 µg or more 6 hours to O/N. Increase enzyme units for shorter digestion time

Molecular Biology of Plants

A Laboratory Course Manual

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MAIZE DNA MINIPREP (used for Tomato and Rapeseed)

Stephen L. Dellaporta, Jonathan Wood, James B. Hicks

The topic of this protocol is rapid microscale methods for isolation of plant DNA without the use of ultracentrifugation with CsCl. The DNA produced is of moderately high molecular weight and serves as a satisfactory substrate for most restriction endonucleases and is suitable for genomic blot analysis. In addition to the rapidity and convenience of minipreps which permit a large number of samples to be processed in just a few hours, the small amount of tissue required (less than 1.0 grams) allows for molecular analysis of plants at a very young stage. Miniprep DNA yields from leaf tissue of most species tested to date are typically 50-100 ug per gram tissue, greater than 50 kb, and remarkably uniform from sample to sample.

The first miniprep procedure we reported for maize DNA isolation (Dellaporta et al, Maize Genetics Cooperation Newsletter, 1983) was adapted from a procedure commonly used for yeast DNA preparation (Davis et al., 1980). Since this report, numerous personal communications have demonstrated that the miniprep procedure or a modification thereof, can be applied to most plant species tested. For example, the method has been successfully used on Nicotiana tabacum N. plumbaginifolium, N. sylvestris, Lyscopericum sp., Amaranthus sp., Glycine max, Petunia hybrida. Several modifications have been applied by these investigators and in our own laboratory in order to extend the application of miniprep procedures to other plant species. The selection of a particular protocol depends to a large degree on the plant species used. However, the procedure reported here was selected to be suitable for most situations.

Procedure

1. Weigh 1 gm of leaf tissue, quick freeze in liquid nitrogen and grind to a fine powder in a 3" mortar and pestle. Transfer powder with liquid nitrogen into a 30 ml Oak Ridge tube.

   It is imperative not to let the tissue thaw once frozen until buffer is added and not to cap the tubes while nitrogen is evaporating.

2. Add 15 ml of Extraction Buffer (EB): 100 mM Tris pH 8, 50 mM EDTA pH 8, 500 MM NaCl, 10 MM mercaptoethanol.

   For maximum DNA yields, the cells are further broken by grinding the mixture at a low setting (about 3) with a Polytron (Brinkmann Instruments, Inc.). However, this step is optional.

3. Add 1.0 ml of 20% SDS, mix thoroughly by vigorous shaking, and incubate tubes at 65°C for 10 min.

[redacted]

4. Add 5.0 ml 5 M potassium acetate. Shake tube vigorously and incubate 0° for 20 min.

   Most proteins and polysaccharides are removed as a complex with the insoluble potassium dodecyl sulfate precipitate.

5. Spin tubes at 25,000 x g for 20 min. Pour supernatant through a Miracloth filter (Calbiochem) into a clean 30 ml tube containing 10 ml isopropanol. Mix and incubate tubes at -20° for 30 min.

6. Pellet DNA at 20,000 x g for 15 min. Gently pour off supernatant and lightly dry pellets by inverting the tubes on paper towels for 10 min.

7. Redissolve DNA pellets with 0.7 ml of 50 mM Tris, 10 MM EDTA, pH 8. Transfer the solution to an Eppendorf tube. Spin the tubes in a microfuge for 10 min to remove insoluble debris.

8. Transfer the supernatant to a new Eppendorf tube and add 75 ul 3M sodium acetate and 500 ul isopropanol. Mix well and pellet the clot of DNA for 30 sec in a microfuge. Wash pellet with 80% ethanol, dry, and redissolve in 100 ul 10 mM Tris, 1 mM EDTA, pH 8.

   Precipitation for 0.3 M sodium acetate using relatively small amounts of isopropanol (about 0.6 volumes) has been reported to separate high molecular weight DNA from polysaccharides (Marmur, 1961). The sodium acetate also yields a tight fibrous precipitate that is easily washed and dried. The DNA will dissolve readily if allowed to rehydrate at 4°C for 1 hr followed by light vortexing.

Minipreps can be stored for several months without evidence of degradation and can be cut with a variety of restriction enzymes and ligated without further purification. We find that 10.0 ul of miniprep DNA is sufficient for a single 8 mm lane in an agarose gel which is to be used for filter hybridization with single-copy probes. Heat-treated RNAase must be added to the restriction reaction to digest contaminating RNA in each prep. Hence, a typical reaction would contain the following:

Miniprep DNA	10.0 ul
10X Restriction Buffer	3.0 ul
0.5 mg/ml RNAase	2.0 ul
Eco RI	8 units
dH20 to 30 ul.	

Digestion is usually complete after 3 hours at 37°C. Occasionally, minipreps are difficult to digest with certain enzymes. This problem can be overcome by adding 5.0 ul of 0.1 M spermidine to the entire miniprep before digestion (see Focus 4(3):12, 1982).

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2. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. 3:208-218.

APPENDIX EA-3

Field Trial Data for FLAVR SAVR™ Tomato

1. Seed germination. To test the hypothesis that genetically engineered lines were unchanged in regards to seed germination, rate and frequency of germination were measured in three field trials, comparing FLAVR SAVR tomatoes with control lines (Table 1). These germination measurements were conducted in the greenhouse prior to transplanting into the field. If the engineered lines were different than the controls, it would be expected that all lines would be in one class separate from the controls. However, this was not the case and no meaningful differences were measured.

Eleven separate tests were conducted consisting of transgenic lines and the control lines from which they were derived. For four of the comparisons, the transgenic line had a higher germination rate and frequency than the control. For five of the comparisons, the controls were higher. For two, the final germination frequencies were the same. An analysis of variance was done using the SAS procedure CATMOD (Table 2). These results have high chi-square values for differences among all lines ("Line"), between transformed and control lines ("Trans"), among the three different time-points ("Day"), among different transgenic lines ("Line*Trans"), and among all lines in regards to rate ("Line*Day"). These differences are a result of very large sample sizes (22,816 transgenic seeds and 5,532 controls) and need to be considered as such, since very large sample sizes allow minor differences to appear significant. Examination of the raw data (Tables 1 and 2) suggests that the large chi-square values are based on variation in rank among the lines: sometimes the transgenics are higher and sometimes lower. For example, in the San Joaquin trial, the transgenic line 501 had a final, average germination frequency of 66.6% as compared to a higher frequency for the 501 control, 85.9%. This ranking changed in the Indio trail, with a frequency of 97.6% for 501 and 71.9% for the control. Also, some of the transgenic lines, such as 501-1019-4 and 501-1035-4 (San Joaquin trial), had very low germination frequencies which resulted in a very high chi-square value (2277.25) for line differences in the analysis of variance (Table 2, Part B). Because of the changes in rank among lines, there is no evidence that the transgenic lines have an increase (or decrease) in germination frequency.

The chi-square value was low for differences in rate between transgenic and controls lines ("Trans*Day"), which provides strong support that germination rate is unaffected by transformation.

Strict quality control (QC) standards (e.g., isolation from other tomatoes to maintain seed purity, cleaning equipment to prevent pollen spread, separate handling of fruit to prevent seed mixing) are implemented for seed production of commercial lines, since uniform, high germination frequency is essential for commercial cultivars. Tomato lines under product development are not finished varieties and therefore

are not subject to strict QC measures such as high quality field production and screening/gravity table or size sorting. Seed production for FLAVR SAVR tomato lines has generally been in the greenhouse which results in lower quality seed at times. The plant breeding and product development process will eliminate or improve lines with slow germination rate and low frequency. Under commercial settings, all seed must have fast germination and a high frequency. Thus, as expected the germination frequency means of the commercial lines were higher than the means of the transgenic lines under development.

Based on these germination results and observations made during eight field trials, FLAVR SAVR tomato seed is not different from seed produced through traditional breeding and is unaffected by the FLAVR SAVR gene and other inserted genes. There is no indication that the transgenic seed will be dispersed differently, last longer or be more competitive in new environments. Therefore, there is no greater potential for FLAVR SAVR tomato or any other tomato cultivar crossed with it to become a weed pest risk, than for traditionally bred tomatoes.

Table 1. Comparison of Seed Germination between FLAVR SAVR and Control Tomatoes from Three Field Trials.

A. Germination Results from Dixon Trial, 5/16/91 Planting (USDA APHIS Permit #91-050-01).

VARIETY	# Cells Planted	GERMINATION					
		7 DAY Count	%	10 DAY	%	14 DAY	%
UC82B1436-25	232	205	88.4%	212	91.4%	228	98.3%
UC82B1436-33	112	24	21.4%	94	83.9%	99	88.4%
UC82B Total	**344**	**229**	**66.6%**	**306**	**89.0%**	**327**	**95.1%**
UC82B CONTROL	**256**	**243**	**94.9%**	**242**	**94.5%**	**250**	**97.7%**
SS 1436-23	128	97	75.8%	99	77.3%	102	79.7%
SS 1436-41	128	85	66.4%	88	68.8%	89	69.5%
SS 1436-59	112	80	71.4%	87	77.7%	92	82.1%
SS 1436-64	112	74	66.1%	79	70.5%	85	75.9%
SS 1436-66	128	93	72.7%	103	80.5%	116	90.6%
SS 1436-67	128	81	63.3%	87	68.0%	94	73.4%
SS Total	**736**	**510**	**69.3%**	**543**	**73.8%**	**578**	**78.5%**
SS Control	**128**	**34**	**26.6%**	**36**	**28.1%**	**36**	**28.1%**
Total Transgenics	**1080**	**739**	**68.4%**	**849**	**78.6%**	**905**	**83.8%**
Total Controls	**384**	**277**	**72.1%**	**278**	**72.4%**	**286**	**74.5%**
Other Controls*							
PSM38179	256	236	92.2%	239	93.4%	246	96.1%
PSX 77884	256	227	88.7%	247	96.5%	255	99.6%
PSX 77684	256	230	89.8%	245	95.7%	251	98.0%
PSX 77384	256	197	77.0%	215	84.0%	244	95.3%
PSM36579	256	96	37.5%	210	82.0%	226	88.3%
Cavalier	256	231	90.2%	237	92.6%	249	97.3%
Monte Carlo	208	161	77.4%	194	93.3%	197	94.7%
Celebrity	256	158	61.7%	242	94.5%	255	99.6%
Merced	256	225	87.9%	235	91.8%	249	97.3%
Santiago	256	198	77.3%	209	81.6%	230	89.8%
Tango	163	152	93.3%	156	95.7%	156	95.7%
Blazer	256	184	71.9%	209	81.6%	238	93.0%
Regency	256	224	87.5%	234	91.4%	253	98.8%
Teresa	256	228	89.1%	236	92.2%	253	98.8%

HMX 6796	232	201	86.6%	205	88.4%	211	90.9%
HMX 8813	128	61	47.7%	115	89.8%	125	97.7%
HMX 7803R	256	231	90.2%	239	93.4%	254	99.2%
Jackpot	88	69	78.4%	73	83.0%	75	85.2%
Sweepstakes	88	77	87.5%	79	89.8%	86	97.7%
Count Fleet	88	66	75.0%	70	79.5%	76	86.4%
Keno	111	91	82.0%	95	85.6%	95	85.6%
Royal Flush	128	122	95.3%	123	96.1%	127	99.2%
Sunny	256	209	81.6%	245	95.7%	253	98.8%
BHN 2	256	244	95.3%	246	96.1%	250	97.7%
BHN 22	256	246	96.1%	247	96.5%	251	98.0%
BHN 95	256	244	95.3%	251	98.0%	254	99.2%
E-686	640	581	90.8%	610	95.3%	621	97.0%
E-695	640	522	81.6%	585	91.4%	597	93.3%
100	256	218	85.2%	229	89.5%	241	94.1%
102	256	242	94.5%	250	97.7%	255	99.6%
103	241	229	95.0%	229	95.0%	235	97.5%
104	256	101	39.5%	123	48.0%	123	48.0%
105	256	232	90.6%	236	92.2%	241	94.1%
106	256	221	86.3%	233	91.0%	243	94.9%
107	256	166	64.8%	175	68.4%	184	71.9%
108	256	179	69.9%	184	71.9%	197	77.0%
109	236	225	95.3%	231	97.9%	234	99.2%
110	245	187	76.3%	196	80.0%	216	88.2%
111	256	253	98.8%	253	98.8%	256	100.0%
113	256	170	66.4%	179	69.9%	188	73.4%
504	256	241	94.1%	247	96.5%	253	98.8%
520	256	213	83.2%	229	89.5%	243	94.9%
ROMA-1	640	622	97.2%	630	98.4%	630	98.4%
HYB.882(ROMA)	256	238	93.0%	243	94.9%	243	94.9%
VICKIE	256	236	92.2%	241	94.1%	252	98.4%
N 73	256	171	66.8%	174	68.0%	182	71.1%
N 75	216	149	69.0%	171	79.2%	176	81.5%
N 791	256	128	50.0%	191	74.6%	215	84.0%
N89	256	217	84.8%	228	89.1%	248	96.9%
HEALANI	120	104	86.7%	108	90.0%	118	98.3%
LONG TOM.	128	97	75.8%	112	87.5%	123	96.1%
Totals for Other Controls	12,788	10,550	82.5%	11,383	89.0%	11,873	92.8%

*Other controls were included in CATMOD analysis only for this trial because the SS control was so low.

[redacted]

B. Germination Results from San Joaquin Trial, 6/10/91 Planting (USDA APHIS Permit #91-107-04).

VARIETY	# Cells Planted	Germination					
		7-DAY Count	%	10 DAY	%	14 DAY	%
501-1001-6	256	170	66.4%	180	70.3%	181	70.7%
501-1001-11	256	178	69.5%	178	69.5%	178	69.5%
501-1001-12	256	189	73.8%	194	75.8%	204	79.7%
501-1001-15	256	88	34.4%	197	77.0%	203	79.3%
501-1019-2	256	126	49.2%	188	73.4%	191	74.6%
501-1019-3	256	157	61.3%	170	66.4%	180	70.3%
501-1019-4	256	77	30.1%	91	35.5%	97	37.9%
501-1019-6	256	147	57.4%	157	61.3%	169	66.0%
501-1019-9	256	171	66.8%	185	72.3%	192	75.0%
501-1035-3	256	111	43.4%	170	66.4%	174	68.0%
501-1035-4	256	98	38.3%	102	39.8%	107	41.8%
Total for 501	**2816**	**1512**	**53.7%**	**1812**	**64.3%**	**1876**	**66.6%**
501 CONTROL	**128**	**108**	**84.4%**			**110**	**85.9%**
502-2021-6	256	212	82.8%	219	85.5%	226	88.3%
502-2021-12	256	172	67.2%	178	69.5%	182	71.1%
502-2021-16	256	158	61.7%	162	63.3%	167	65.2%
502-2037-9	256	239	93.4%	241	94.1%	243	94.9%
502-2047-3	256	239	93.4%	247	96.5%	247	96.5%
502-2047-4	256	233	91.0%	240	93.8%	240	93.8%
502-2047-10	256	241	94.1%	244	95.3%	244	95.3%
502-2047-12	256	240	93.8%	244	95.3%	248	96.9%
502-2047-15	256	245	95.7%	248	96.9%	250	97.7%
502-2052-8	256	231	90.2%	234	91.4%	237	92.6%
502-2057-6	256	224	87.5%	237	92.6%	238	93.0%
502-2070-2	256	241	94.1%	245	95.7%	246	96.1%
502-2070-3	128	118	92.2%	118	92.2%	121	94.5%
502-2070-6	256	210	82.0%	212	82.8%	212	82.8%
502-2092-5	256	221	86.3%	239	93.4%	241	94.1%
502-2092-16	256	225	87.9%	241	94.1%	244	95.3%
Total for 502	**3968**	**3449**	**86.9%**	**3549**	**89.4%**	**3586**	**90.4%**
502 CONTROL	**128**	**110**	**85.9%**			**112**	**87.5%**

CR3-610-1	256	227	88.7%	236	92.2%	243	94.9%
CR3-610-9	256	144	56.3%	144	56.3%	153	59.8%
CR3-639-6	256	171	66.8%	183	71.5%	184	71.9%
CR3-652-14	256	243	94.9%	246	96.1%	247	96.5%
CR3-652-5	256	235	91.8%	238	93.0%	238	93.0%
CR3-652-7	256	234	91.4%	240	93.8%	242	94.5%
CR3-663-5	256	204	79.7%	216	84.4%	221	86.3%
CR3-613-1	256	214	83.6%	222	86.7%	223	87.1%
CR3-613-6	256	215	84.0%	224	87.5%	221	86.3%
CR3-613-9	256	189	73.8%	195	76.2%	196	76.6%
CR3-613-10	256	236	92.2%	236	92.2%	237	92.6%
CR3-613-11	256	236	92.2%	239	93.4%	242	94.5%
CR3-613-12	256	229	89.5%	235	91.8%	236	92.2%
CR3-614-9	256	225	87.9%	231	90.2%	230	89.8%
CR3-614-15	256	203	79.3%	215	84.0%	219	85.5%
CR3-623-7	256	229	89.5%	238	93.0%	238	93.0%
CR3-623-9	256	231	90.2%	232	90.6%	234	91.4%
CR3-638-5	88	62	70.5%	62	70.5%	69	78.4%
CR3-644-2	256	237	92.6%	238	93.0%	242	94.5%
CR3-644-5	256	214	83.6%	215	84.0%	219	85.5%
CR3-644-16	208	179	86.1%	182	87.5%	186	89.4%
CR3-663-10	256	202	78.9%	220	85.9%	220	85.9%
CR3-623-19	256	243	94.9%	250	97.7%	250	97.7%
Total for CR3	**5672**	**4802**	**84.7%**	**4937**	**87.0%**	**4990**	**88.0%**
CR3 CONTROL	**256**			**248**	**96.9%**		
CR5-855-5	256	161	62.9%	190	74.2%	202	78.9%
CR5-863-4	128	110	85.9%	118	92.2%	122	95.3%
CR5-863-19	256	202	78.9%	221	86.3%	227	88.7%
CR5-814-7	256	234	91.4%	234	91.4%	234	91.4%
CR5-814-17	256	226	88.3%	239	93.4%	240	93.8%
Total for CR5	**1152**	**933**	**81.0%**	**1002**	**87.0%**	**1025**	**89.0%**
CR5 CONTROL	**256**			**236**	**92.2%**		
Total Transgenics	**13608**	**10696**	**78.6%**	**11300**	**83.0%**	**11477**	**84.3%**
Total Controls	**768**	**218**	**85.2%**	**484**	**94.5%**	**222**	**86.7%**

C. Seed Germination Counts for Indio Trial, 1/3/92 Planting (USDA APHIS Permit #91-268-01).

VARIETY	# Cells Planted	GERMINATION					
		7 Day Count	%	10 Day Count	%	14 Day Count	%
501-1001-15	384	359	93.5%	369	96.1%	382	99.5%
501-1001-11	384	339	88.3%	351	91.4%	374	97.4%
501-1001-12	384	307	79.9%	336	87.5%	359	93.5%
501-1019-6	384	340	88.5%	340	88.5%	384	100.0%
501 Total	**1536**	**1345**	**87.6%**	**1396**	**90.9%**	**1499**	**97.6%**
501 CONTROL	**384**	**117**	**30.5%**	**231**	**60.2%**	**276**	**71.9%**
502-2021-16	384	301	78.4%	348	90.6%	384	100.0%
502-2037-9	384	361	94.0%	373	97.1%	384	100.0%
502-2047-4	384	379	98.7%	382	99.5%	384	100.0%
502-2052-8	384	379	98.7%	382	99.5%	384	100.0%
502-2092-5	384	377	98.2%	377	98.2%	384	100.0%
502 Total	**1920**	**1797**	**93.6%**	**1862**	**97.0%**	**1920**	**100.0%**
502 CONTROL	**384**	**308**	**80.2%**	**334**	**87.0%**	**384**	**100.0%**
CR5-863-19	**384**	**320**	**83.3%**	**348**	**90.6%**	**381**	**99.2%**
CR5 CONTROL	**384**	**294**	**76.6%**	**319**	**83.1%**	**347**	**90.4%**
CR3-613-9	384	338	88.0%	364	94.8%	384	100.0%
CR3-613-12	384	367	95.6%	367	95.6%	384	100.0%
CR3-623-19	384	333	86.7%	369	96.1%	384	100.0%
CR3-644-5	384	340	88.5%	371	96.6%	384	100.0%
CR3 Total	**1536**	**1378**	**89.7%**	**1471**	**95.8%**	**1536**	**100.0%**
CR3 CONTROL	**384**	**261**	**68.0%**	**309**	**80.5%**	**384**	**100.0%**

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CR3-610-1	384	343	89.3%	367	95.6%	384	100.0%
SS 1436-23-6	384	287	74.7%	306	79.7%	352	91.7%
SS 1436-23-13	128	125	97.7%	126	98.4%	126	98.4%
SS 1436-23-25	384	342	89.1%	353	91.9%	382	99.5%
SS 1436-23-34	128	95	74.2%	109	85.2%	110	85.9%
SS 1436-38-6	128	81	63.3%	95	74.2%	103	80.5%
SS 1436-38-12	128	48	37.5%	85	66.4%	93	72.7%
SS 1436-40-2	128	89	69.5%	103	80.5%	112	87.5%
SS 1436-40-11	64	29	45.3%	44	68.8%	49	76.6%
SS 1436-64-4	384	310	80.7%	310	80.7%	321	83.6%
SS 1436-64-11	384	333	86.7%	375	97.7%	383	99.7%
SS 1436-64-19	384	300	78.1%	328	85.4%	334	87.0%
SS 1436-64-25	128	111	86.7%	115	89.8%	128	100.0%
SS Total	**2752**	**2150**	**78.1%**	**2349**	**85.4%**	**2493**	**90.6%**
SS CONTROL	**512**	**406**	**79.3%**	**439**	**85.7%**	**512**	**100.0%**
Total Transgenics	**8128**	**6990**	**86.0%**	**7426**	**91.4%**	**7829**	**96.3%**
Total Controls	**2048**	**1386**	**67.7%**	**1632**	**79.7%**	**1903**	**92.9%**

D. Combined Germination Results from the Three Field Trials: Dixon, San Joaquin and Indio.

VARIETY	# Cells Planted	GERMINATION					
		7 DAY Count	%	10 DAY	%	14 DAY	%
Total Transgenics	**22,816**	**18,425**	**80.8%**	**19,575**	**85.8%**	**20,211**	**88.6%**
Total Controls*	**2,638**	**1,881**	**71.3%**			**2,411**	**91.4%**
Total Controls*	**2,894**			**2,394**	**82.7%**		

*The San Joaquin trial did not have measurements for each time point, so the total number of cells planted was unequal. Does not include the "other controls" from the Dixon trial.

Table 2. Analysis of Seed Germination Rates and Frequency for FLAVR SAVR and Control Tomatoes. Data analyzed using CATMOD on SAS.

A. Germination Results from Dixon Trial, 5/16/91 Planting (USDA APHIS Permit #91-050-01).

Analysis of Variance Table*

Source	DF	Chi-Square	Prob
Intercept	1	5142.87	0.0001
Trans	1	296.11	0.0001
Day	2	683.64	0.0001
Residual	2	0.82	0.6648

*Other controls were included in CATMOD analysis only for this trial because the SS control was so low.

B. Germination Results from San Joaquin Trial, 6/10/91 Planting (USDA APHIS Permit #91-107-04).

Analysis of Variance Table

Source	DF	Chi-Square	Prob
Intercept	1	750.79	0.0001
Line	4	2277.25	0.0001
Trans	1	27.49	0.0001
Day	2	26.58	0.0001
Line*Trans	2*	41.04	0.0001
Line*Day	8	20.63	0.0082
Trans*Day	1*	1.53	0.2157
Residual	1	0.16	0.6855

Note: Effects marked with * contained 1 or more singularities (i.e., redundant parameters).

C. Seed Germination Counts for Indio Trial, 1/3/92 Planting (USDA APHIS Permit #91-268-01).

Analysis of Variance Table

Source	DF	Chi-Square	Prob
Intercept	1	2655.23	0.0001
Line	4	262.59	0.0001
Trans	1	298.57	0.0001
Day	2	317.73	0.0001
Line*Trans	4	362.31	0.0001
Line*Day	6*	34.23	0.0001
Trans*Day	2	9.31	0.0095
Residual	10	30.75	0.0006

Note: Effects marked with * contained 1 or more singularities (i.e., redundant parameters).

[redacted]

2. Characterization of the FLAVR SAVR tomato. FLAVR SAVR tomatoes were characterized in regards to molecular, physical, genetic and agronomic qualities based on results from field, greenhouse and laboratory experiments. The following Tables 3-9 summarize these results (Kramer et al. 1992; Kramer et al. 1990, Redenbaugh et al. 1992). Tables 4 and 5 show that there were no differences between transgenic and nontransgenic tomatoes in regards to nutritional and tomatine levels. Tables 6-9 indicate that transgenic tomatoes were changed only for characteristics that are related to the altered trait of reduced levels of polygalacturonase. No changes were observed that were related to the *kan*^{r} gene or APH(3')II, except for the expected resistance to kanamycin.

Table 3. Molecular Characterization of Eight Lines of FLAVR SAVR Tomatoes.

Component	Quantitation
Number of *kan*^{r} genes (haploid)	≤3
Levels of APH(3')II	<0.08% of total protein
Number of FLAVR SAVR genes (haploid)	≤3
Level of PG mRNA	<10% of control lines
Level of PG enzyme activity	<1% of control lines
Gene linkage between *kan*^{r} and FLAVR SAVR genes	yes
Number of insertion sites	one

Table 4. Nutritional Components (RDAs) for Eight pCGN1436 Lines, Five Controls as Compared to Normal Ranges for Tomato.

Constituent	Normal range	Measured range for pCGN1436 lines	Measured range for control lines
Protein	0.85 g (.015 se)	0.75 - 1.14	0.53 - 1.05
Vitamin A	192 - 1667 IU	330 - 1600	420 - 2200
Vit. B_1 (Thiamin)	16 - 80 µg	38 - 72	39 - 64
Vit. B_2 (Riboflavin)	20 - 78 µg	24 - 36	24 - 36
Vitamin B_6	50 - 150 µg	86 - 150	10 - 140
Vitamin C	8.4 - 59 mg	15.3 - 29.2	12.3 - 29.2
Nicotinic acid (Niacin)	0.3 - 0.85 mg	0.43 - 0.70	0.43 - 0.76
Calcium	4.0 - 21 mg	9 - 13 mg	10 - 12
Magnesium	5.2 - 20.4 mg	7 - 12	9 - 13
Phosphorus	7.7 - 53 mg	25 - 37	29 - 38
Sodium	1.2 - 32.7 mg	2 - 5	2 - 3
Iron	0.2 - 0.95 mg	0.2 - 0.41	0.26 - 0.42

Table 5. Tomatine Levels in Green and Red Fruit of FLAVR SAVR Tomatoes (Construct pCGN1436).

Fruit Stage	FLAVR SAVR Range	Control Range
Green	124.7-860.8µg/g dwt	58.6-999.2 µg/g dwt
Red	1.54-7.59	0.70-7.17

Table 6. Comparison of Selected Tomatoes Containing the FLAVR SAVR Gene with Nontransformed Controls.

Component	Changed	Unchanged
Recommended Daily Allowances		+
Potential toxins		+
Taste		+
Serum viscosity	+	
Other processing traits		+
Horticultural traits		+
Fungal resistance	+	
Color (pigmentation)		+
Softening rate	+	

[redacted]

Table 7. Morphological Components of FLAVR SAVR Tomatoes as Compared to Controls.

Component	Changed	Unchanged
Fruit color		+
Fruit size		+
Fruit shape		+
Fruit firmness after harvest	+	
Other morphological characters		+

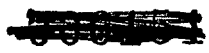
Table 8. Processing Components of FLAVR SAVR Tomatoes as Compared to Controls.

Component	Changed	Unchanged
Total solids	+	
Soluble solids	+	
pH		+
Acidity		+
Color (lycopene		+
Serum viscosity	+	
Consistency	+	

Table 9. Disease Resistance of FLAVR SAVR Tomatoes as Compared to Controls.

Component	Changed	Unchanged
Fruit weight loss	+	
Fruit area with lesions	+	
Lesion size	+	

These data demonstrate that FLAVR SAVR tomatoes were altered for very specific traits that are predictable from the function of the polygalacturonase gene and the effect of decreasing its activity by 99%. It all areas measured, unintended effects were not found. These data and observations suggest that breeding and development of FLAVR SAVR tomatoes are directly analogous to traditional tomato breeding methods, and that FLAVR SAVR tomatoes pose no greater risk than other tomato cultivars.



Additional Characterization of the FLAVR SAVR tomato: APH(3')II, PG, vitamins A & C levels and molecular stability. The following information is provided to address the issues of phenotypic variability. These data have been generated on two transformants, designated CR3-613 and CR3-623, and independently derived via *Agrobacterium*-mediated transformation of tomato variety CR3 (see Figure 1 for CR3 pedigree). The eight transgenic tomato lines (see Figure 2 for 501 pedigree) for which data were previously generated (Redenbaugh et al. 1992) did not include these two CR3 transgenic lines. However, the CR3 lines went through the same selection process at the same time as the eight lines described in the FLAVR SAVR™ submission (see Appendix D-1 of Redenbaugh et al. 1992). CR3-613 and CR3-623 represent two of the 11 CR3 plants noted in Table 3 of Appendix D-1 in Redenbaugh et al. (1992) as having progeny that segregate 3:1 for the *kan*^{r} gene. CR3-613-9 and CR3-613-12 represent individual T_2 progeny of the original CR3-613 plant that was identified as homozygous at the T-DNA locus. Similarly, CR3-623-19 is an individual, homozygous offspring of the original CR3-623 plant.

A. Expression Level of APH(3')II

Samples of ripe fruit from individual T_4 and T_5 plants were subjected to Western analysis and the results are shown in Figures 3 and 4. One gram of ripe fruit tissue was ground to a powder in liquid N_2 and added to 2 ml of boiling sample buffer (0.125M Tris, pH 6.8, 5% SDS, 20% glycerol and 0.05% bromophenol blue). β-Mercaptoethanol was added to a final concentration of 5% and equal volumes of each sample (75 µl) were run on 11.25% polyacrylamide gels along with purified APH(3')II spiked into control samples and molecular weight markers. Proteins were blotted to nitrocellulose and visualized by reaction with APH(3')II antiserum, goat anti-rabbit IgG anti-bodies coupled to alkaline phosphatase and Western Blue™ color reagent. Fruit from a nontransformed control CR3 plant also analyzed had no detectable signal for the APH(3')II protein (lane 5, Fig 3 and lane 7, Fig 4), as expected. Addition of 10 ng of purified APH(3')II to the CR3 control protein sample (lane 2, Fig 3 and lane 3, Fig 4) and 2 ng of purified APH(3')II to the CR3 control protein sample (lane 3, Fig 3 and lane 5, Fig. 4) however, could easily be detected by the procedure. Samples of fruit from fourth and fifth generation CR3-613 plants (lanes 6-14, Fig 3) gave signals representing significantly less than 2 ng of APH(3')II or less than 0.07 µg of APH (3')II protein per gram of fresh fruit tissue. Tomato fruit is 5% solids and 7% of the solids are protein. Therefore, 1 gram of fruit tissue contain 3.5 mg of protein and APH(3')II represents significantly less than 0.002% of the total fruit protein in the CR3-613 line. Samples of fruit from fourth and fifth generation CR3-623 plants (lanes 8-11, Fig. 4) gave signals about equal to or higher than 2 ng of APH(3')II (lane 5, Fig. 4) but less than 10 ng of purified APH(3')II or less than 0.35 µg of APH(3')II protein per gram of fresh fruit tissue. This higher limit represents significantly less than 0.01% of the total fruit protein in the CR3-623 line.

Some variability between individual homozygous plants in APH(3')II levels is evident within particular generations. There appears to be less APH(3')II in the

fruit from the individual fifth generation CR3-623-19 plants represented by lanes 10 and 11 than in the plant represented by lane 9 on Fig. 4, for example. The difference observed between lane 8 and lanes 10 and 11 on Fig. 4 also appears to reflect variability between fourth and fifth generation CR3-623-19 plants in fruit levels of APH(3')II.

The variation observed likely represents a combination of: 1) the normal phenotypic variability observed for most tomato traits, due in large part to differences in micro- and macro-environmental conditions in the field and the consequences of those differences on general gene function; and 2) variation inherent in Western blot analysis methods. In consideration of the observed variability, conservative estimates have been made for APH(3')II levels in the FLAVR SAVR tomatoes examined here (compare lanes 3, 8 and 9 in Figure 4, for example).

B. Expression Levels of PG

The direct effect of expression of the FLAVR SAVR™ gene is a reduction in PG protein levels. Comparison of PG protein levels and PG enzyme activity levels in fruit from individual fourth and fifth generation transformed plants and in control plant fruit was demonstrated using SDS-PAGE analysis and a PG enzyme activity assay, respectively.

SDS-PAGE Analysis

The same protein extracts used in Western analysis were also subjected to SDS-PAGE analysis (see Expression Level of APH(3')II). 75 µl aliquots of each sample were separated by PAGE on 11.25% SDS polyacrylamide gels. To visualize total proteins, gels were stained with 0.5% Coomassie Brilliant Blue R in a solution of 10% acetic acid and 25% isopropanol, followed by destaining with 10% acetic acid containing 5% methanol.

The results of this analysis are shown in Figure 5. The PG protein band is the intensely staining band migrating slightly faster than the 46 kd protein in the lane containing the molecular weight standards (compare lanes 2 and 1, Fig. 5). PG protein levels are high in protein samples extracted from control fruit (lanes 2 and 12, Fig. 5) and highly reduced in protein samples extracted from the FLAVR SAVR™ fruit from the CR3-613 transgenic tomato line. Some apparent, minor variability in PG protein levels may be evident in Figure 5.

PG Enzyme Activity

PG enzyme activities were determined using the Extraction Procedure 1 and the PG assay method described in Appendix D-4 (Redenbaugh et al. 1992). The results of this analysis are shown in Table 10. Numbers representing PG activity levels in control CR3 fruit vary from 0.95-1.2. Numbers representing PG activity levels in the

[redacted]

offspring of the CR3 transformants appeared to vary somewhat between plants in the same generation (0.18-0.31 in T_4 generation CR3-613 plants; 0.18-0.26 in T_4 generation CR3-623 plants; 0.09-0.22 in T_5 generation CR3-613 plants). From these numbers it is also apparent that PG activity levels in the offspring of the CR3-613 transformant vary somewhat between generations of homozygous plants. At least part of this variation is due to variability in the assay itself as results from replicate assays carried out on the same fruit typically vary over 0.09 A_{540} units (see Table 10). Overall, the variation within or between generations observed among the homozygous offspring of the CR3 transformants is no greater than the variation observed from the CR3 controls.

C. Nutrients Vitamin A and Vitamin C.

Measurements of compounds for which recommended daily allowances (RDAs) have been determined, including vitamins A and C, were performed by the National Food Laboratory, Dublin, CA, using standard methods as described in Appendix D-6 (Redenbaugh et al. 1992). These standard measurements were made on ripe fruit samples from fourth and fifth generation transformed plants and from control plants. The results of this analysis are shown in Table 11.

Major changes in nutritive composition were not observed in either fourth or fifth generation FLAVR SAVR™ tomatoes (see Table 11). All of the vitamins and minerals measured during this analysis were within the normal ranges observed for these components in tomato fruit (see Table E.3, Redenbaugh et al. 1992) with the exceptions noted below.

The vitamin C value for the single sample of fourth generation CR3-623 fruit was 15% lower than the normal range. Vitamin C measurements on the three fifth generation fruit samples of this line were all well within the normal range. Additionally, Vitamin C measurements conducted on multiple samples of fourth generation (9 samples) and fifth generation (17 samples) CR3-623 fruit for a shelf-life study (see attached report) were all within the normal range for this vitamin in tomato fruit.

The calcium level measured in the single sample of fourth generation fruit from the CR3-613 was 20% lower than values in the normal range for tomato fruit. Again, the calcium level measured in six additional samples of fruit from this line (fifth generation) were all within the normal range for tomatoes.

The vitamin B6 levels were somewhat higher in CR3 control fruit (8% higher mean value) and in fifth generation fruit from one of the FLAVR SAVR™ lines (14% higher mean value). All three of the control samples and four of the six of the CR3-613 fruit samples tested had vitamin B6 levels higher than the normal range previously observed in tomatoes. These results probably reflect a genetic propensity of the CR3 tomato variety to have higher than "normal" vitamin B6 levels.

D. Molecular Stability of the Transferred DNA

The molecular stability of the transferred DNA in two independently derived lines of FLAVR SAVR™ tomatoes is described in the attached report entitled "Stable Integration of Inserted DNA."

[redacted]

Table 10. PG Enzyme Activities.

T1 generation[a, b]		T4 generation		T5 generation[a,b]	
Genotype[c]	A 540	Genotype[c]	A 540	Genotype[c]	A 540
CR3 control	1.26(3) [1.14-1.35]	CR3 control	1.17	CR3 control	1.09(10) [.95-1.2]
CR3 1436-613-A	0.34	CR3 1436-613-9-1A	0.19	CR3 1436-613-9-1	0.14
CR3 1436-613-B	0.68	CR3 1436-613-9-1B	0.28	CR3 1436-613-9-2	0.2
CR3 1436-613-C	0.49	CR3 1436-613-9-2A	0.2	CR3 1436-613-9-3	0.18
CR3 1436-613-D	0.42	CR3 1436-613-9-2B	0.23	CR3 1436-613-9-4	0.11
		CR3 1436-613-9-3A	0.3	CR3 1436-613-9-5	0.12
		CR3 1436-613-9-3B	0.22	CR3 1436-613-9-6	0.12
		CR3 1436-613-9-4A	0.26	CR3 1436-613-9-7	0.09
		CR3 1436-613-9-4B	n.d.	CR3 1436-613-9-8	0.22
				CR3 1436-613-9-9	0.22
		CR3 1436-613-12-1A	0.31	CR3 1436-613-9-10	0.15
		CR3 1436-613-12-1B	0.23		
		CR3 1436-613-12-2A	0.18		
		CR3 1436-613-12-2B	0.27		
		CR3 1436-613-12-3A	0.25		
		CR3 1436-613-12-3B	n.d.		
		CR3 1436-613-12-4A	0.29		
		CR3 1436-613-12-4B	0.26		
CR3 1436-623-A	0.25	CR3 1436-623-19-1A	0.24		
CR3 1436-623-B	0.29	CR3 1436-623-19-1B	0.2		
CR3 1436-623-C	0.25	CR3 1436-623-19-2A	0.18		
CR3 1436-623-D	0.19	CR3 1436-623-19-2B	0.21		
		CR3 1436-623-19-3A	0.22		
		CR3 1436-623-19-3B	0.21		
		CR3 1436-623-19-4A	0.21		
		CR3 1436-623-19-4B	0.26		

[a] PG activity in control fruit of T1 and T5 generations represents the mean of multiple measurements, numbers in ( ) indicate N.

[b] Values in [ ] represent range of activity measurements.

[c] CR3 indicates the tomato variety; 1436 indicates that the plant was transformed with pCGN1436; additional numbers indicate individual plants; letters indicate independent assays of fruit from a particular plant.

TABLE 11A. Nutritional Profile Analysis of fruit from fourth generation FLAVR SAVR™ tomatoes.

DIXON*

Nutrient	Genotype: CR3 Control	CR3-613	CR3-623
Protein (%)	0.875	0.91	0.79
Vitamin A (IU)	520	520	680
Vitamin C (mg/100g)	8.69	12.47	7.18
Calcium (mg/100g)	5	3.2	6.9
Iron (mg/100g)	< 0.25	<0.25	<0.25
Sodium (mg/100g)	4.5	4	4.6
Thiamin (mg/100g)	0.04	0.04	0.04
Riboflavin (mg/100g)	0.02	0.02	0.02
Niacin (mg/100g)	0.38	0.56	0.71
Magnesium (mg/100g)	10	9	10
Phosphorus (mg/100g)	22.9	21	18.3
Vitamin B6 (mg/100g)	128	127	100

* Fruit material was harvested from a field trial conducted near Dixon, CA during Summer 1992. Non-control material represents fourth generation transformants.

TABLE 11B. Nutritional Profile Analysis of fruit from fifth generation FLAVR SAVR™ tomatoes.

MANTECA**

Nutrient	CR3 Control		CR3-613-9†	
Protein (%)	1.14, 0.97, 0.81	(0.97 ± 0.17)	0.98,0.99,1.06	(1.01 ± 4.0)
Vitamin A (IU)	1200,1270,1100	(1190 ± 85)	1080,1240,1020	(1110 ± 113)
Vitamin C (mg/100g)	22.7,20.0,21.9	(21.5 ± 1.4)	22.3,22.3,16.1	(20.2 ± 3.6)
Calcium (mg/100g)	7.6,11,12	(10.2 ± 2.8)	12,9.2,9.4	(10.2 ± 1.6)
Iron (mg/100g)	0.31,0.34,0.59	(0.41 ± 0.15)	0.35,0.36,0.37	(0.36 ± 0.01)
Sodium (mg/100g)	8.7,6.7,4.9	(6.8 ± 1.9)	6.9,8.1,6.5	(7.2 ± 0.83)
Thiamin (mg/100g)	0.03,0.02,0.02	(0.02 ± 0.01)	0.02,0.02,0.02	(0.02 ± 0.00)
Riboflavin (mg/100g)	0.03,0.04,0.03	(0.03 ± 0.01)	0.05,0.03,0.03	(0.04 ± 0.01)
Niacin (mg/100g)	0.52,0.43,0.43	(0.46 ± 0.05)	0.54,0.46,0.52	(0.51 ± 0.04)
Magnesium (mg/100g)	14.0,12.1,10.1	(12.1 ± 2.0)	11.7,12.0,13.3	(12.3 ± 0.85)
Phosphorus (mg/100g)	22.7,23.7,19.3	(21.9 ± 2.3)	28.9,20.7,24.8	(24.8 ± 4.1)
Vitamin B6 (mg/100g)	151,160,179	(163 ± 14)	189,159,164	(171 ± 16)

Nutrient	CR3-613-12†		CR3-623-19†	
Protein (%)	1.37,0.93,1.16	(1.15 ± 0.22)	1.11,0.92,1.07	(1.03 ± 0.08)
Vitamin A (IU)	583,821,597	(667 ± 134)	768,935,995	(899 ± 118)
Vitamin C (mg/100g)	14.7,20.0	(17.4 ± 3.8)	15.5,19.3,17.0	(17.3 ± 1.9)
Calcium (mg/100g)	10,11,8.6	(9.9 ± 1.2)	9.4,9.3,9.1	(9.3 ± 0.22)
Iron (mg/100g)	0.35,0.24,0.27	(0.29 ± 0.06)	0.3,0.27,0.31	(0.29 ± 0.02)
Sodium (mg/100g)	9.3,7,6.3	(7.5 ± 1.6)	8.1,8.6,8.7	(8.5 ± 0.32)
Thiamin (mg/100g)	0.02,0.02,0.03	(0.02 ± 0.01)	0.02,0.02,0.03	(0.02 ± 0.01)
Riboflavin (mg/100g)	0.03,0.03,0.03	(0.03 ± 0.00)	0.03,0.04,0.03	(0.03 ± 0.01)
Niacin (mg/100g)	0.50,0.48,0.54	(0.51 ± 0.03)	0.48,0.67,0.46	(0.54 ± 0.12)
Magnesium (mg/100g)	16.0,14.7,10.0	(13.6 ± 3.2)	12.4,9.7,12.0	(11.4 ± 1.5)
Phosphorus (mg/100g)	31,22,25	(26 ± 4.6)	20.5,23.7,16.8	(20.3 ± 3.5)
Vitamin B6 (mg/100g)	123,166,129	(139 ± 23)	117,152,127	(132 ± 18)

** Fruit material was harvested from a field trial conducted in Manteca, CA during Summer and Fall 1992. Non-control material represents fifth generation transformants.

† The additional numbers associated with CR3-613 and CR3-623 indicate the numbers of the individual homozygous T_2 progeny plant selected for further development. CR3-613-9 and CR3-613-12 are therefore both representative of advanced generations of the original CR3-613 transformant.

Figure 1. Pedigree/Genealogy CR3 tomato cultivars.

Line CR3 was transformed in 1990 producing the two new transformed lines, CR3-613 and CR3-623, which were planted in the greenhouse September 1990. CR3-613 and CR3-623 are different transformation events of CR3.

September 1990	= T_1 plants from lab to Greenhouse
December 1990	= T_2 seed produced in Galt greenhouse, CA
February 1991	= T_2 seed planted in Indio, CA = T_2 backup seed planted in Galt greenhouse, CA
June 1991	= T_3 seed harvested out of Indio, CA = T_3 seed harvested out of Galt greenhouse, CA
July 1991	= T_3 seed from Galt planted in Manteca, CA
October 1991	= T_4 seed harvested out of Manteca, CA
February 1992	= T_4 seed from Manteca planted in Indio, CA
May 1992	= T_4 seed from Manteca planted in Dixon, CA
June 1992	= T_5 seed harvested out of Indio, CA
June 1992	= T_5 seed from Indio planted in Manteca, CA
Aug.-Sept. 1992	= T_5 seed harvested out of Dixon, CA
Oct.-Nov. 1992	= T_6 seed harvested out of Manteca

[redacted]

Figure 2. Pedigree/Genealogy PGI 501 tomato cultivars.

Line 501 was transformed in 1990 producing the several new transformed lines, including 501-1001, which were planted in the greenhouse May 1990 (Lines 501 and CR3 are not related).

1/23/90 - tomato line PGI 501 transformed in Calgene laboratory at 1920 5th street, Davis, CA (Lab notebook S952 018 page 80).

↓

T1 tomato plantlets produced containing ASPG gene.

↓

5/4/90 - plantlets transplanted into Davis greenhouse, then subsequently transplanted to Galt greenhouse.

↓

T2 seed produced summer 1990 in greenhouse.

↓

11/27/90 - T2 seed planted in Davis greenhouse, then subsequently transplanted to Galt greenhouse.

↓

T3 seed produced winter 1990/1991 in greenhouse.

↓

6/10/91 - T3 seed planted in greenhouse.

↓

7/15/91 - T3 plants transplanted to San Joaquin field trial. Late-season pesticide application: 8/26/91 - Dyrene; and 99/3/91 - Bravo and Cygon mix.

↓

T4 seed produced summer 1991 in field.

↓

10/28/91 - T4 seed harvested.

↓

11/4/91 - T4 seeds planted in Davis greenhouse #8.

↓

12/3/91 - T4 plants subsequently transplanted to Galt greenhouse. Pesticide application: 1/22/92 - Pyrenone and Maverick; 1/28 - Benlate and Dithane; 3/30 - Malathion and Pyrenone.

↓

2/10/92 - flowers allowed to self for fruit production. Fruit to be harvested for toxicity studies.

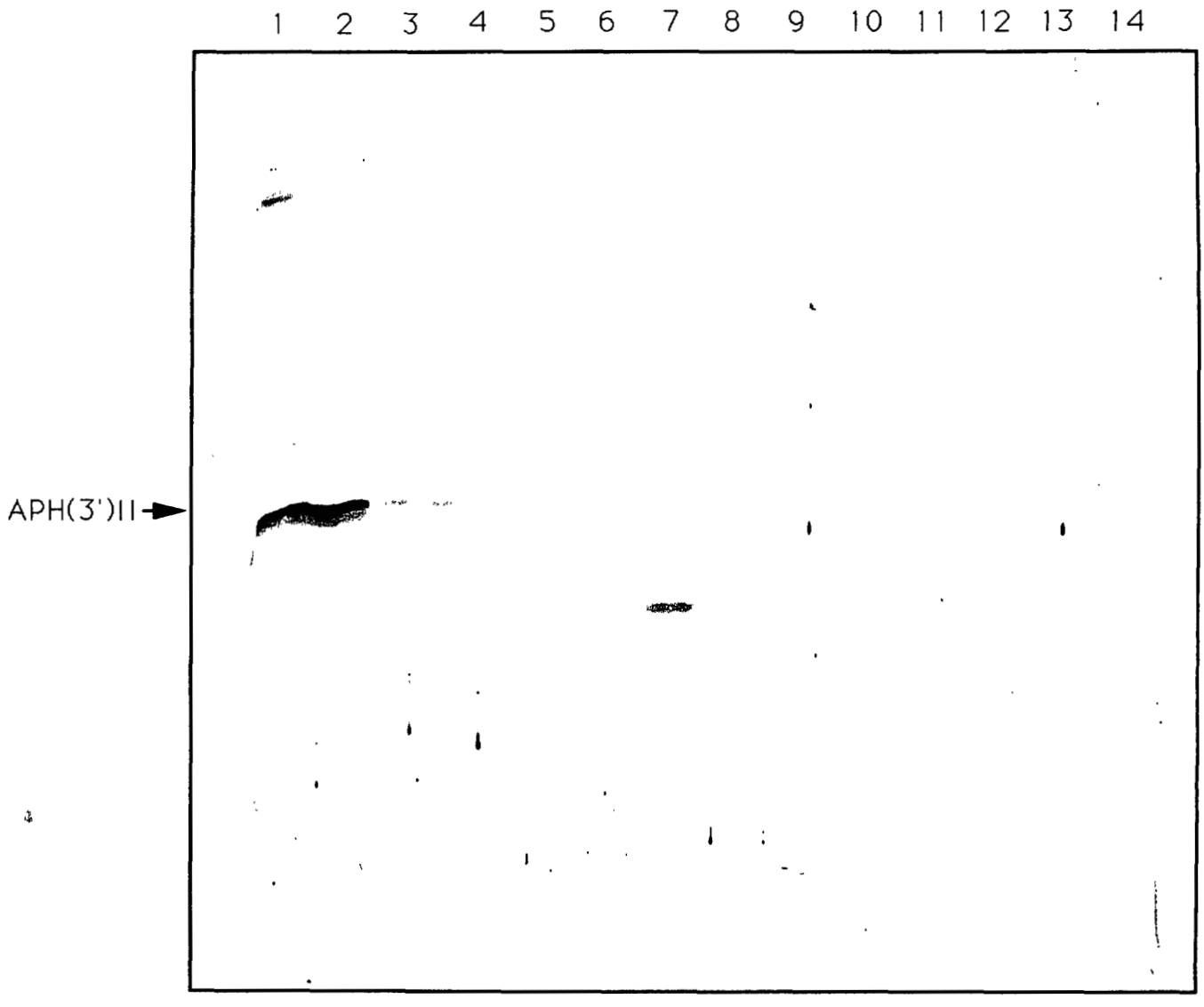


Figure 3: Samples were prepared as described; 75 µl protein aliquots were subjected to Western analysis. Lane designations are as follows: 1 - Rainbow molecular weight markers with 2 ng purified APH(3')II, 2 - CR3 control with 10 ng purified APH(3')II, 3 - CR3 control with 2 ng purified APH(3')II, 4 - Blank, 5 - CR3 Control, 6 - CR3 613-12-1 T_4, 7 - CR3 613-12-2 T_4, 8 - CR3 613-12-3 T_4, 9 - CR3 613-9-1 T_5, 10 - CR3 613-9-2 T_5, 11 - CR3 613-9-3 T_5, 12 - CR3 613-12-1 T_5, 13 - CR3-613-12-2 T_5, 14 - CR3 613-12-3 T_5. The APH(3')II antibody (catalog #5307-511721 lot CC161A) and APH(3')II protein used as standards (catalog #5306-639134, lot CD212A) were obtained from 5 Prime -> 3 Prime, Inc. (5603 Arapahoe, Boulder, CO).

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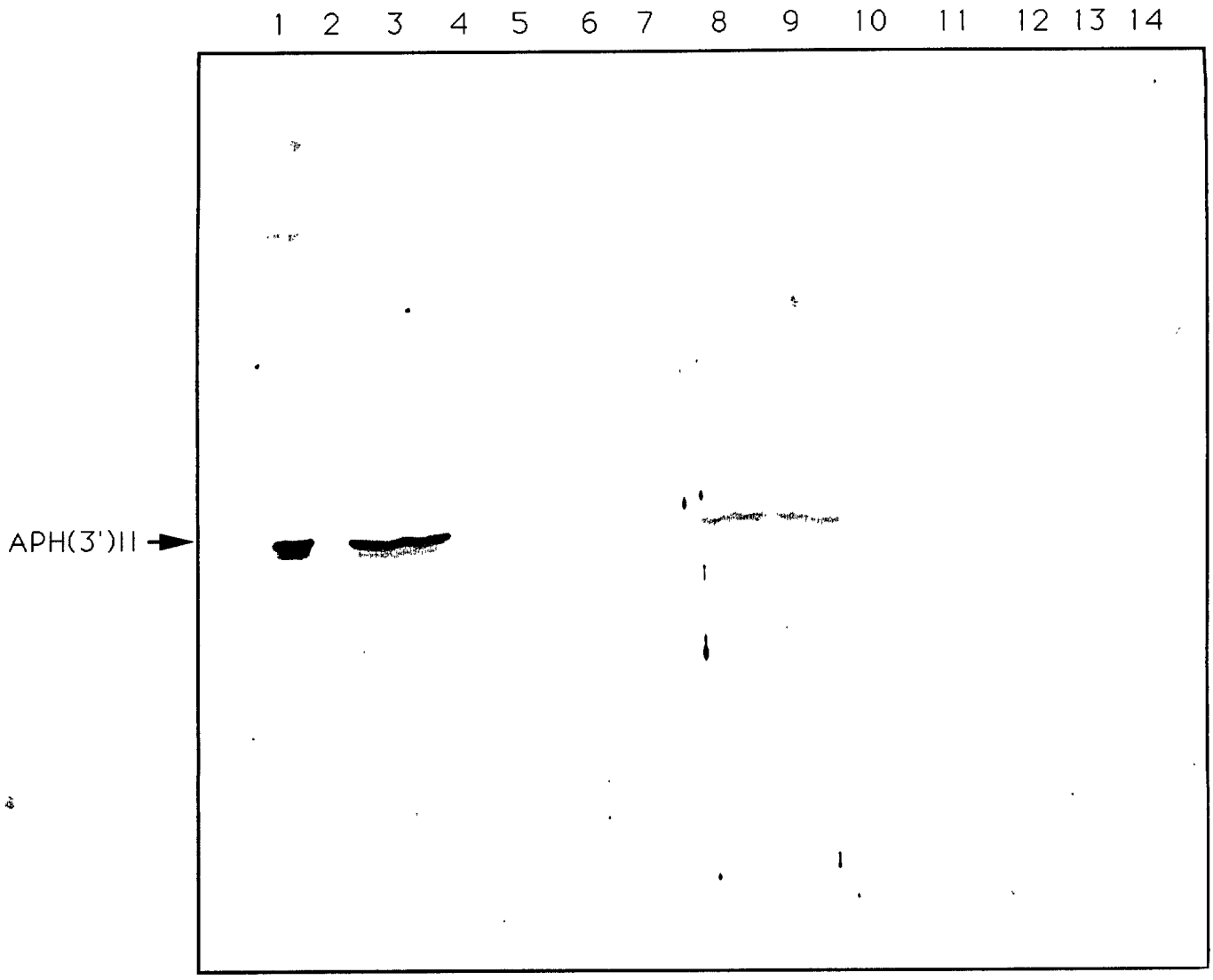

Figure 4: Samples were prepared as described: 75 µl protein aliquots were subjected to Western analysis. Lane designations are as follows: 1 - Rainbow molecular weight markers with 10 ng purified APH(3')II, 2 - Blank, 3 - CR3 control with 10 ng purified APH(3')II, 4 - Blank, 5 - CR3 control with 2 ng purified APH (3')II, 6 - Blank, 7 - CR3 control, 8 - CR3 623-19-1 T_4, 9 - CR3 623-19-1 T_5, 10 - CR3 623-19-2 T_5, 11 - CR3 623-19-3 T_5, 12 - 14 Blank. The APH(3')II antibody (catalog #5307-511721 lot CC161A) and APH(3')II protein used as standards (catalog #5306-639134, lot CD212A) were obtained from 5 Prime -> 3 Prime, Inc. (5603 Arapahoe, Boulder, CO).

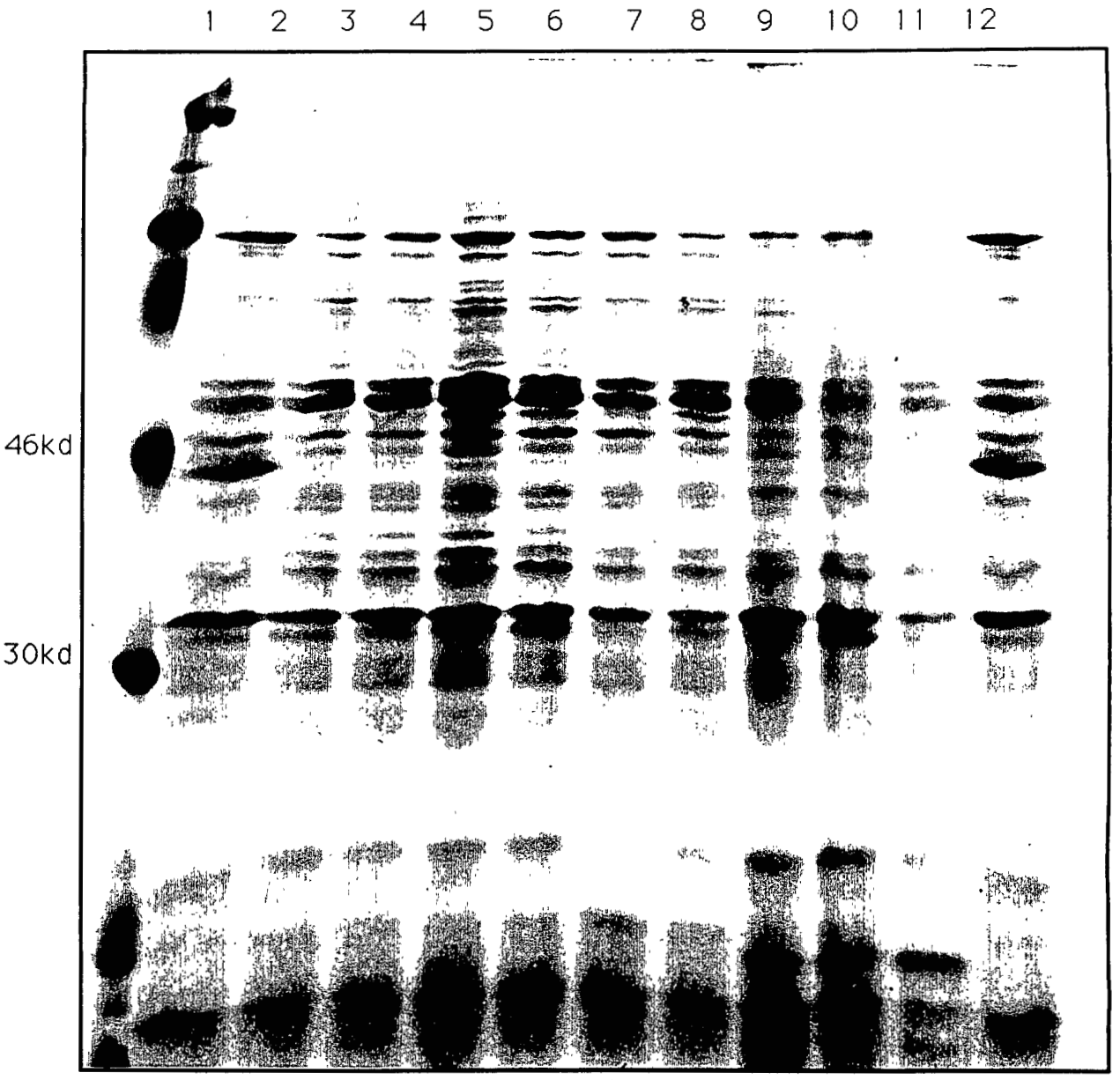

Figure 5: Samples were prepared as described: 75 µl aliquots were subjected to SDS-PAGE analysis. Lane designation are as follows: 1- Rainbow molecular weight markers, 2 - CR3 control, 3 - CR3 613-12-3 T_5, 4 - CR3 613-12-2 T_5, 5 - CR3 613-12-1 T_5, 6 - CR3 613-9-3 T_5, 7 - CR3 613-9-2 T_5, 8 - CR3 613-9-1 T_5, 9 - CR3 613-12-3 T_4, 10 - CR3 613-12-2 T_4, 11 - CR3 613-12-1 T_4, 12 - CR3 control.

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Stable Integration and Structure of Inserted DNA

INTRODUCTION

The structures of the T-DNA region of pCGN1436 that had been inserted into the genomes of eight example transgenic tomato lines were characterized in a previous document (FLAVR SAVR™ Tomato: Status as Food. Request for Advisory Opinion, U.S. Food and Drug Administration, Docket 391A-0330/API, August 12, 1991). Evidence of the genetic stability of the inserted T-DNA, based on a seed germination assay (see Appendix D-3, Docket 391A-0330) and DNA analyses of progeny plants (see Appendix D-2, Docket 391A-0330), over at least two generations was also presented. In this report the DNA analysis is extended to cover four generations of transgenic plants in line 501-1001 and five generations of transgenic plants in line CR3-613. The descendants of these two original transformants were produced as the results of "selfed" crosses.

ANALYTICAL RESULTS

Evidence of the genetic stability of the T-DNA inserted into the genomes of plants 501-1001 and CR3-613 were obtained through analysis of the ability of these lines to maintain a specific DNA fragment consisting of a border between the inserted T-DNA region and the tomato DNA. This analysis was accomplished using the Southern blot technique (Southern 1975) as described (see Docket 391A-0330). Genomic DNA was isolated from a nontransformed plant of the variety 501, the original transgenic 501-1001 T1 plant, an offspring resulting from self pollination the 501-1001 plant (501-1001-15 T2), a selfed progeny of the 501-1001-15 T2 plant (501-1001-15 T3) and a 501-1001-15 T3 progeny (501-1001-15 T4)(Figures 1 and 2). Similarly, DNA was isolated from a nontransformed plant of the CR3 variety, the original CR3-613 T1 plant and T3 and T5 homozygous descendants (Figures 3 and 4). The isolated DNA was cut with restriction endonucleases BamHI and BglII, subjected to electrophoresis through a 0.7% agarose gel

and transferred to a nylon membrane using capillary action. After transfer the Southern blot was hybridized with a radiolabeled DNA fragment representing the coding region of the *kan*^{r} gene (*kan*^{r} gene probe, see Appendix D-2, Docket 391A-0330). As shown in Figure 1, a hybridization signal corresponding to a DNA fragment of approximately 9 kb is observed in the lane containing DNA from transformant 501-1001 just as it was in the experiment depicted in Figure 10, Appendix D-2 of Docket 391A-0330. A DNA fragment of approximately 9 kb is also observed in the lanes containing each of the three 501-1001 descendants; no hybridization signal is observed in the lane containing DNA isolated from the non-transformed 501 line, as expected (see Figure 1). Similarly, as shown in Figure 3, a hybridization signal corresponding to a DNA fragment of approximately 5 kb is observed in the lanes containing DNA from the original CR3-613 transformant and from its descendants. (The designations CR3-613-9 and CR3-613-12 refer to individual homozygous offspring from the original transformed CR3-613 plant; as such they represent the same T-DNA insertion event.) Again, no hybridization signal is observed in the lane containing DNA isolated from the nontransformed CR3 line, as expected (see Figure 3).

Additional evidence of the genetic stability of the T-DNA insertions in lines 501-1001 and CR3-613 was obtained through analysis of the ability of these lines to maintain two copies and four copies, respectively, of the FLAVR SAVR gene once homozygosity at the T-DNA locus was established in the T2 generation. This analysis was also accomplished using the Southern blot technique as previously described (Docket 391A-0330; Sanders *et al.* 1992). After transfer, Southern blots prepared as described above were hybridized with a radio labeled DNA fragment representing the coding region of the PG gene (the 390 bp PG gene probe, see Appendix D-2 Figure 2, Docket 391A-0330). This double-stranded probe hybridizes to DNA fragments produced from both the introduced FLAVR SAVR gene and the endogenous PG gene. As shown in Figure 2, both the 1036 bp DNA fragment (representing the two copies of the endogenous PG gene) and the 967 bp DNA fragment (representing the introduced FLAVR SAVR gene) are observed in each of

the DNA samples isolated from T2, T3 and T4 generation descendants of the 501-1001 plant. The two fragments in each of the DNA samples appear to hybridize to the PG probe with equal intensity as would be expected if two copies of the FLAVR SAVR gene were present in the genomes of the 501-1001 plants tested. As shown in Figure 4, the same two fragments in the DNA sample from the CR3-613 T1 plant appear to hybridize to the PG probe with equal intensity, as would be expected if two copies of the FLAVR SAVR gene were present in the genome of this T1 individual. The FLAVR SAVR gene fragment appears to hybridize to the PG probe with twice the intensity as the endogenous PG gene fragment in lanes 3, 4 and 5 of Figure 4, as would be expected if four copies of the FLAVR SAVR gene were present in the genomes of the fourth and fifth generation plants tested. The higher molecular weight DNA fragment observed probably represents another DNA sequence in the tomato genome that shares some homology with the PG gene as it is also present in samples of DNA isolated from non-transformed control plants (see Appendix D-2 Figure 3, Docket 391A-0330, for example).

DISCUSSION OF METHODS AND ANALYTICAL RESULTS

The structure of the inserted T-DNA region, and thereby the number of integrated FLAVR SAVR and *kan*^{r} genes in the plant's genome, has been previously elucidated for transformant 501-1001 (see Fig. D-1, Docket 391A-0330). This particular plant has one complete copy of the T-DNA region from pCGN1436 inserted in its genome at one locus. The T-DNA locus in the 501-1001 T1 parent plant was also shown, using a germination assay on kanamycin, to segregate among progeny 501-1001 T2 plants as expected based on Mendelian genetics (see Appendix D-2 Table D-1 and Figure 20, Docket 391A-0330). In this report the Southern analysis on 501-1001 plants has been extended to document the stability of the T-DNA locus over two more sexual generations. This report also documents stability, over five sexual generations, of the T-DNA locus in an additional pCGN1436 transformant, CR3-613, derived from tomato variety CR3.

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Progeny plant 1001-15 was deemed homozygous based on the seed germination assay (see Appendix D-3, Docket 391A-0330) and the DNA analysis shown in Figure 2. Similar analyses of CR3-613 progeny resulted in the determination that 613-9 and 613-12 individuals were homozygous. The 967 bp DNA fragment representing the antisense PG gene and the 1036 bp DNA fragment representing the endogenous PG gene hybridize to the PG gene probe with equal intensity in the DNA sample from plant 1001-15. Since the hybridization intensity of the 967 bp antisense PG gene fragment in the original hemizygous 501-1001 plant was approximately half that of the 1036 bp endogenous PG gene fragment, this result indicates that the 501-1001-15 plant carries two copies of the FLAVR SAVR gene, one on each of two homologous chromosomes, and is therefore homozygous at the T-DNA insert locus. The homozygous states of 501-1001 T2 progeny plants 1001-6, 1001-11 and 1001-12 were similarly determined (see Appendix D-2 Table D-1 and Figure 20, Docket 391A-0330). Since the hybridization intensity of the 967 bp antisense PG gene fragment in the original hemizygous CR3-613 plant was equal to that of the 1036 bp endogenous PG gene fragment, the CR3-613 T1 plant carries two copies of the FLAVR SAVR gene. Since the hybridization intensity of the 967 bp antisense PG gene fragment in the subsequent CR3-613 plant generations was approximately twice that of the 1036 bp endogenous PG gene fragment, these descendants carry four copies of the FLAVR SAVR gene, two on each of two homologous chromosomes, and are therefore homozygous at the T-DNA insert locus.

Once homozygosity has been established at the T-DNA locus, selfed progeny from that homozygous individual should always "breed true"; that is, continue to carry the copy(ies) of the FLAVR SAVR gene on both homologous chromosomes. Thus, in descendants of the original homozygous 1001-15 individual the 967 bp antisense PG gene fragment and the 1036 bp endogenous PG gene fragment should always hybridize to the PG gene probe with equal intensity when subjected to Southern analysis as described above. The experiment depicted in Figure 2 demonstrates this expected hybridization intensity in T3

and T4 generation plants. Therefore, two copies of the introduced FLAVR SAVR DNA are indeed still present in the T3 and T4 generations of plant 501-1001 and, as expected, these plants have remained homozygous. Further, in descendants of the original homozygous 613-9 and 613-12 individuals the antisense PG gene fragment should always hybridize to the PG gene probe with twice the intensity of the endogenous PG gene fragment when subjected to the same analysis. The experiment depicted in Figure 4 demonstrates this expected hybridization intensity in T4 and T5 generation plants.

The experiment depicted in Figure 1 also demonstrates the stability of the T-DNA region in transformant 501-1001 and its descendants. In this case a DNA fragment consisting of a border between the inserted T-DNA region and the plant DNA into which the T-DNA has been integrated (identified using a *kan*^{r} gene hybridization probe) is tracked from the T1 through the T4 generation. Such a border fragment serves as a kind of fingerprint for an individual transgenic plant since for each transformant the T-DNA region has been inserted into a different site in the tomato genome. The "fingerprint" 9 kb border fragment for the original 501-1001 transformant (as well as the border "fingerprint" fragments for seven other tomato plants transformed with pCGN1436) is shown in Figure 10 of Appendix D-2 in Docket 391A-0330, and the "fingerprint" border fragment for the original CR3-613 transformant is shown in Fig. 3 (lane 2) of this document. If a T-DNA region is stably integrated into the genome of a transgenic plant, its signature border fragment(s) and only its signature border fragment(s) should be evident in all of its selfed descendants. As shown in Figure 1, a 9 kb fragment that hybridizes to the *kan*^{r} gene hybridization probe is indeed evident in each of the four 501-1001 plant generations examined. As shown in Figure 3, a 5 kb fragment that hybridizes to the *kan*^{r} gene hybridization probe is indeed evident in each of the three CR3-613 plant generations examined. These results provide strong evidence that the T-DNA region in the original 501-1001 and CR3-613 transformants, and therefore the FLAVR SAVR and *kan*^{r} genes

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contained within those T-DNA regions, have remained stably integrated into the same sites in the tomato genome through three and four subsequent generations, respectively.

SUMMARY

The genetic stability of T-DNA insertions into plant genomic DNA has been well documented (see Fraley *et al.* 1986; Kuhlemeier *et al.* 1987 and references therein). It is therefore expected that once integrated into the tomato genome the T-DNA region of pCGN1436 will behave like any other Mendelian trait. Evidence has been presented here indicating that the structure of the inserted T-DNA from pCGN1436 in transformants 501-1001 and CR3-613 remained unchanged and that the inserted T-DNA locus in each did, in fact, behave predictably, based on Mendelian genetics, over four and five selfed generations, respectively. These data provide specific evidence that the T-DNA region from pCGN1436 is stably integrated into the 501-1001 plant genome. Genetic stability of the T-DNA region is expected in all plants transformed with the pCGN1436 gene construct; the 501-1001 and CR3-613 transformants have been used as simple examples to illustrate these expected results.

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FIGURE LEGENDS

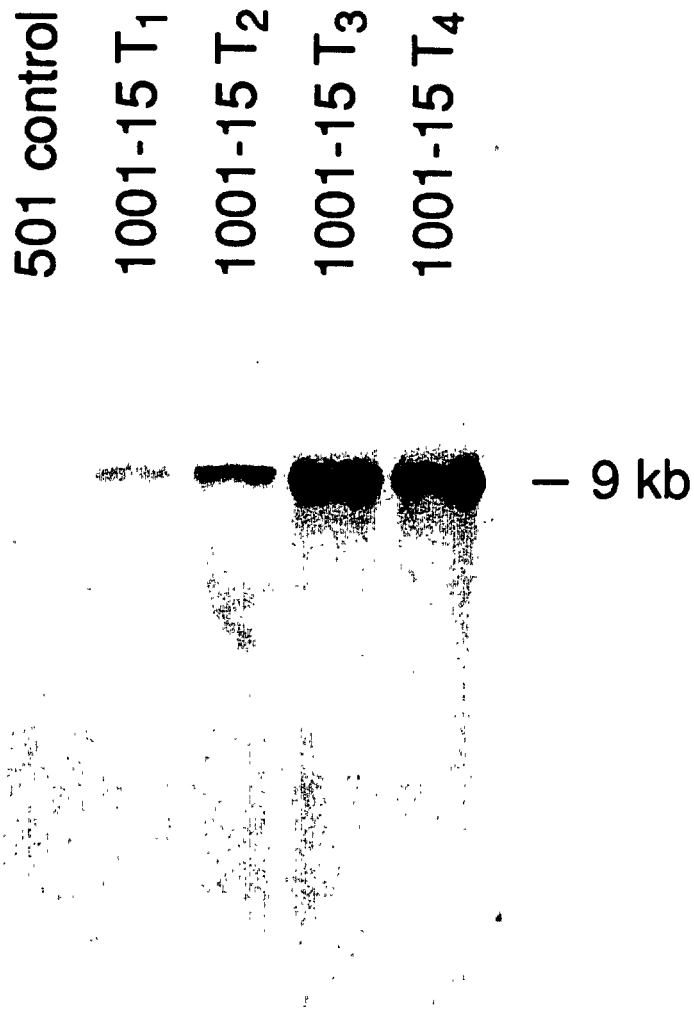
Figure 1: Stability of T-DNA insertion in transformant 501-1001 over four generations. Genomic DNA samples from tissue harvested from each of four generations (as indicated and from nontransformed, control 501 plant) were digested with BamHI and BglII and then run on a 0.7% agarose gel. After transfer to a nylon membrane, the blot was hybridized with a ^{32}P-labeled probe representing the coding region of the *kan^r* gene followed by autoradiography. The approximately 9 kb fragment representing the border between the T-DNA insertion and the plant DNA adjoining the left border of the T-DNA insertions, a fragment characteristic of the 501-1001 transformation event (see Fig. 10, p369 Docket 391A-0330) is apparent in all four samples of genomic DNA isolated from the original transformant and its descendants. This border fragment is not observed in the sample of genomic DNA isolated from the control plant, as expected. Band intensity difference is due to loading of 2-5x more DNA in T_3 and T_4 lanes than in T_1 and T_2 lanes.

Figure 2: Homozygosity of T-DNA insertion in transformant 501-1001 over three generations. Genomic DNA samples from tissue harvested from each of the three 501-1001 generations indicated was processed for Southern blot experiments as described in Figure 1. After transfer to nylon membrane, blots were hybridized with a ^{32}P-labeled probe representing coding region sequences of the PG gene (the 390 bp PG gene probe, see appendix D-2 Figure 2, Docket 391A-0330). The relative intensity of the 967 bp DNA fragment representing the introduced FLAVR SAVR™ gene and the 1036 bp DNA fragment representing the two copies of the endogenous PG gene are the same in each generation as would be expected once the T-DNA region containing the FLAVR SAVR ™ gene became homozygous in the T_2 generation.

Figure 3: Southern blot of DNA from T1, T4 and T5 generations of CR3-613 DNA digested with BamHI and BglII and hybridized with the *kan*^{r} gene probe. Lane assignments are as follows: (1) CR3 control, (2) CR3-613 T1, (3) CR3-613-9 T4, (4) CR3-613-9 T5 (5) CR3-613-12 T4 and (6) CR3-613-12 T5.

Figure 4: Southern blot of DNA from T1, T4 and T5 generations of CR3-613 DNA digested with BamHI and BglII and hybridized with the PG gene probe. Lane assignments are as follows: (1) CR3 control, (2) CR3-613 T1, (3) CR3-613-9 T4, (4) CR3-613-9 T5 and (5) CR3-613-12 T5.

Figure 1:



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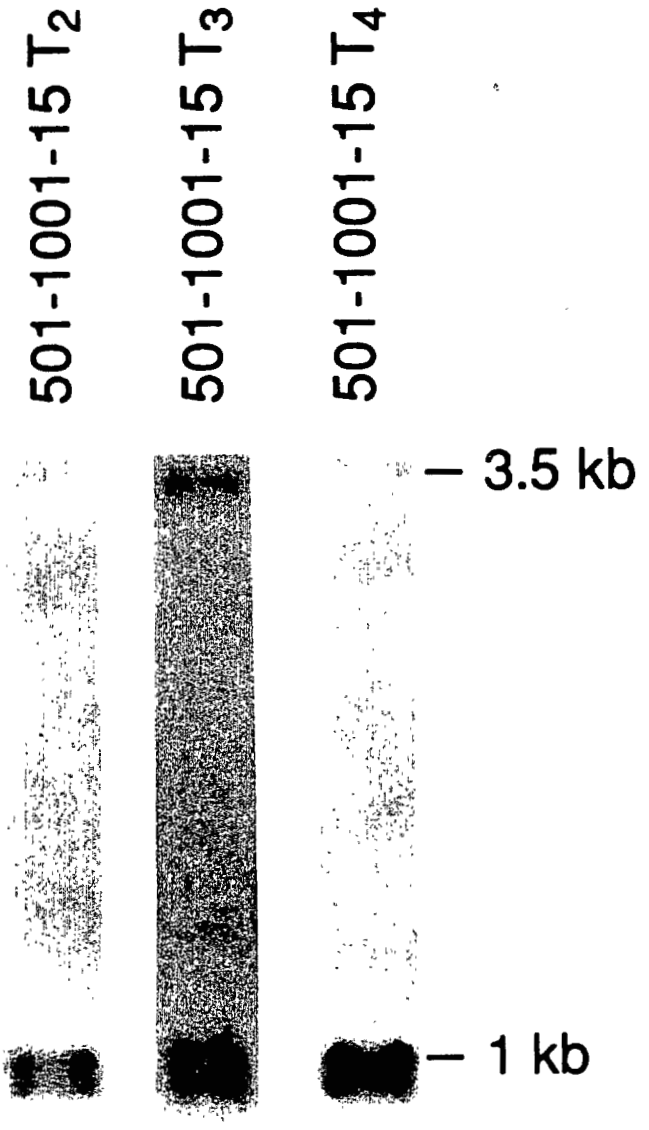

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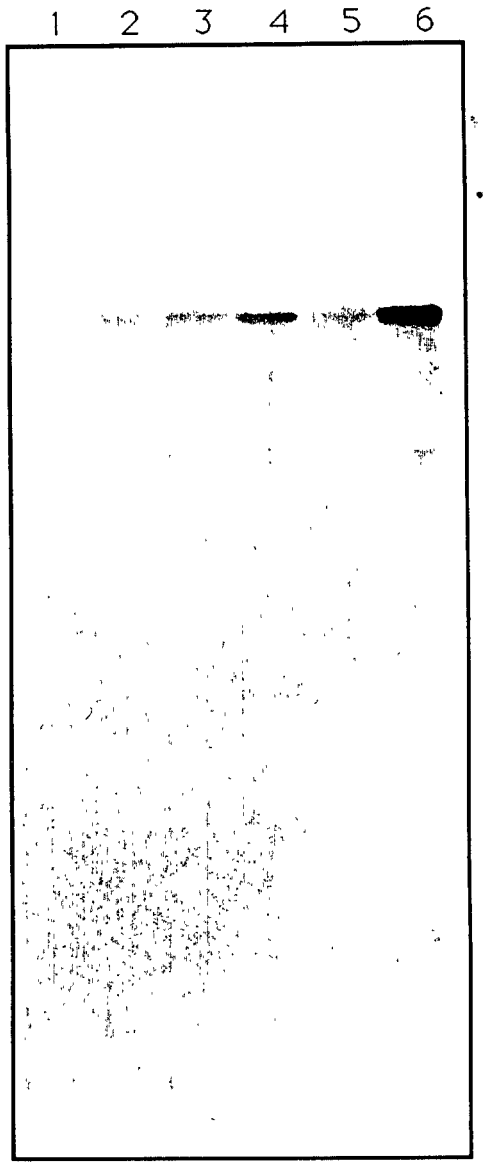

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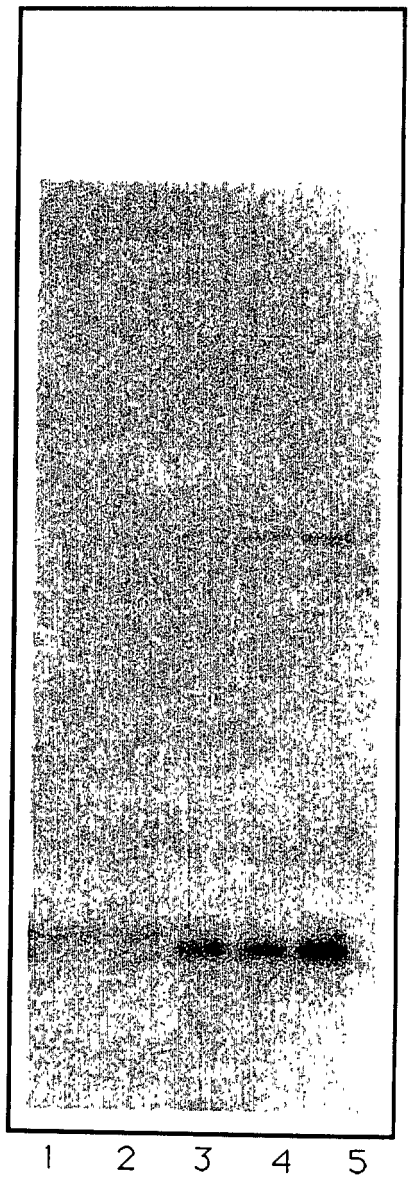
Figure 4:



Vitamin Levels in FLAVR SAVR™ Tomatoes During Storage

Experimental Design

Three lines of mature green and pink FLAVR SAVR tomatoes (613-9, 613-12 and 623-19) were harvested from the Manteca, CA field trial on October 5, 1992 (Table 1). FLAVR SAVR tomatoes will be harvested at various stages from mature green to pink. The pink stage is the "vine-ripe" stage and represents the maximum age at which the tomatoes will be harvested. These fruit are fifth generation (T_5) transformants. Mature green and pink fruit were also harvested on the same day from the progenitor, nontransformed tomato variety CR3. Three replicate samples of fruit from each line were prepared for analysis by the National Food Laboratory (NFL) on October 9. Each sample replication throughout this study consisted of three whole fresh tomatoes from the October 5th harvest (Table 1).

The mature green fruit were then placed in an environment of ethylene gas (approx. 100 ppm) for 48 hrs. This treatment follows standard procedures presently used with commercial gassed green tomatoes. Three replicate samples of fruit from each line were then prepared for analysis by the NFL on October 12 (Table 1).

The remainder of the harvested fruit, consisting of fruit picked green and gassed and fruit picked pink, were stored in cardboard boxes at 56°C. Additional replications of the pink fruit were prepared for analysis by the NFL on October 16 and October 23. Additional replications of the gassed green fruit were prepared for analysis by the NFL on October 23 and October 30 (Table 1).

For the final analysis, the fruit had reached the end of its shelf life. This end point was determined by a physical examination of the fruit for texture, firmness and general appearance.

Table 1. Experimental design.

Tomato line	Stage at harvest[a]	Date of 1st analysis	Date of 2nd analysis	Date of 3rd analysis	Date of 4th analysis
CR3-613-9	mature green	10/9[b]	10/12[c]	10/23[c]	10/30[c]
CR3-613-12	mature green	10/9[b]	10/12[c]	10/23[c]	10/30[c]
CR3-623-19	mature green	10/9[b]	10/12[c]	10/23[c]	10/30[c]
CR3 control	mature green	10/9[b]	10/12[c]	10/23[c]	10/30[c]
CR3-613-9	pink	10/9	10/16	10/23	[d]
CR3-613-12	pink	10/9	10/16	10/23	[d]
CR3-623-19	pink	10/9	10/16	10/23	[d]
CR3 control	pink	10/9	10/16	10/23	[d]

[a]All tomato fruit picked on October 5, 1992.
[b]Fruit not gassed.
[c]Fruit gassed for 48 hours.
[d]Fruit picked pink did not last past the third analysis.

[redacted]

Results

The results of this analysis are shown in Tables 2, 3, 4 and 5.

- Vitamin C levels, for both the gassed green and picked pink fruit, were not significantly different among the genotypes nor over the course of storage life tested.

- All vitamin C levels were within the normal range found in ripe tomatoes (8.4-59 mg/100 g fresh tissue; see Table E.3, p.486, FLAVR SAVR™ Tomato: Status as Food, Request for Advisory Opinion, U.S. Food and Drug Administration, Docket #91A-0330/API, August 12, 1991) with two minor exceptions. One sample replication of the CR3 control fruit picked pink (from the 10/16 sampling date) was approximately 6.5% lower than the normal range (7.87 mg/100 g). However, the other two replications from this time point were within range. One sample replication of the CR3-613 fruit (from the 10/30 sampling date) was 1.8% lower than the normal range (8.25 mg/100 g). The other two replications from this time point were within range.

- Pro-vitamin A (β-carotene) levels for fruit picked green, and fruit picked green and gassed, did change significantly over the course of the study, as expected, in fruit from both control and transgenic plants. As is typical during tomato fruit ripening, levels of pro-vitamin A increased after gassing during storage as the tomatoes turned red. Pro-vitamin A levels in ripened fruit (10/23 and 10/30 sampling dates) were all within the normal range found in ripe tomatoes (192-1667 IU/100g fresh fruit).

- Pro-vitamin A levels for fruit picked pink were all within the normal range found in ripe tomatoes (192-1667 IU/100 g fresh fruit) and were maintained at a normal level throughout storage.

Conclusion

FLAVR SAVR tomatoes, stored to the end of their shelf life, have the same levels of pro-vitamin A and vitamin C as compared to controls or compared to the normal range of these vitamins in other tomato varieties.

[redacted]

TABLE 2. Vitamin C Levels in Fruit Harvested Mature Green (mg/100 g fresh tissue).

	10/9	ave/sd*	10/12	ave/sd	10/23	ave/sd	10/30	ave/sd
CR3 Control	14.7 16.6 20.4	17.2±2.9	16.6 18.1 18.1	17.6±0.9	14.6 14.8 17.3	15.6±1.5	16.1 16.1	16.1±0.0
CR3-613-9	13.6 16.3 14	14.6±1.5	12.5 18.1 11.9	14.2±3.4	10.9 16.9 12.8	13.5±3.1	16.9 9.00	13.0±5.9
CR3-613-12	20.8 14 17	17.3±3.4	22.3 18.9 21.2	18.5±5.7	10.9 19.1 14.6	14.9±4.1	9.00 8.25 11.6	9.6±1.8
CR3-613-19	13.2 21 16.6	16.9±3.9	15.1 14.7 12.1	14.0±1.6	16.5 12.8 14.6	14.6±1.9	11.6 11.6	11.6±0.0

*average ± standard deviation.

TABLE 3. Vitamin A Levels in Fruit Harvested Mature Green (IU/100g fresh fruit).

	10/9	ave/sd*	10/12	ave/sd	10/23	ave/sd	10/30	ave/sd
CR3 Control	208 186 364	253±97	387 346 195	309±101	530 452 440	474±49	644 588	616±40
CR3-613-9	271 291 118	223±95	178 169 230	192±33	337 467 342	382±74	915 519	717±280
CR3-613-12	193 136 173	167±29	293 361 212	289±75	415 342 423	393±45	586 370 488	481±108
CR3-613-19	184 210 92	162±62	152 146 229	176±46	750 654 275	560±251	470 460	465±7

*average ± standard deviation.

[redacted]

TABLE 4. Vitamin C Levels in Fruit Harvested Pink (mg/100 g fresh tissue).

	10/9	ave/sd*	10/16	ave/sd	10/23	ave/sd
CR3 Control	22.3	21.7±1.1	9.0	11.1±4.7	24.8	22.1±3.9
	22.3		16.5		24.0	
	20.4		7.87		17.6	
CR3-613-9	22.3	14.6±2.4	19.9	15.8±1.4	19.9	20.5±7.5
	14.4		13.5		27.8	
	15.7		14.6		24.8	
CR3-613-12	12.9	14.6±2.4	17.3	15.8±1.4	19.5	20.5±7.5
	16.3		14.6		28.5	
			15.4		13.5	
CR3-613-19	14.7	14.7±0.4	18.4	15.2±3.9	16.3	16.8±0.7
	14.4		16.3		16.5	
	15.1		10.9		17.6	

*average ± standard deviation.

TABLE 5. Vitamin A Levels in Fruit Harvested Pink (IU/100g fresh fruit).

	10/9	ave/sd*	10/16	ave/sd	10/23	ave/sd
CR3 Control	646	977±38	582	670±167	657	653±67
	1390		566		584	
	896		863		718	
CR3-613-9	1390	920±408	741	642±130	407	678±237
	660		689		780	
	709		495		846	
CR3-613-12	536	516±32	678	605±165	522	526±65
	479		416		463	
	533		721		592	
CR3-613-19	436	496±123	657	543±131	457	380±100
	414		571		417	
	637		400		267	

*average ± standard deviation.

[redacted]

APPENDIX EA-4

Characterization of the Inserted *kan*^{r} Gene by Southern Blot Analyses

[redacted]

Appendix D-2. Characterization of the Inserted FLAVR SAVR™ and *kan*^{r} Genes by Southern Blot Analyses.

I. FLAVR SAVR and *kan*^{r} Genes.

The T-DNA region of pCGN1436 contains the FLAVR SAVR and selectable marker genes. The structure and number of genes contained in the T-DNA and inserted into the genomes of transgenic tomatoes has been characterized using the technique originally described by Southern (1975) and now commonly referred to as the Southern blot. Intact genomic DNA is isolated from an individual plant, incubated with restriction endonucleases (enzymes that selectively cleave DNA at specific nucleotide sequences), and the resulting fragments are separated by size using electrophoresis through a gel matrix. The DNA fragments in the gel matrix are then denatured and transferred to a solid membrane, often by capillary action (therefore the reference to a "blot"), for ease of subsequent manipulations. The individual single-stranded (denatured) DNA fragments on such a blot are capable of forming double-stranded molecules with complementary (homologous) nucleic acid sequences by DNA:DNA hybridization. Individual sequences can be labelled by chemical or radioactive methods and serve as a hybridization "probe" for detecting and identifying specific, homologous sequences among those found in the plant DNA on the blot.

The type of information derived from a Southern blot experiment depends on the particular enzymes used to cleave the DNA and the sequences of the particular probes used for hybridization. Several different experimental designs of Southern blot experiments have been utilized, in conjunction with genetic analysis, to generate data used to determine the number of FLAVR SAVR and *kan*^{r} genes inserted into representative transgenic tomatoes. The number of genetic loci (at which the T-DNA region from pCGN1436 has been inserted into the tomato genome) has been determined in part by identifying the number of borders between the inserted T-DNA and the adjacent plant DNA. The design of this experiment takes advantage of the known locations of the recognition sites for specific restriction endonucleases within the T-DNA region of pCGN1436. Tomato DNA cut with an enzyme or enzymes known to cut within the T-DNA should contain DNA fragments made up of that part of the T-DNA region between the recognition site for the particular enzyme used and the adjoining plant DNA, as well as the adjoining plant DNA between the T-DNA:plant DNA juncture and the nearest recognition site for that particular enzyme found within the tomato genome. Border fragments can then be identified by using hybridization probes containing DNA sequences from the region of the T-DNA contributing to a particular border fragment. For simple insertions of single T-DNA regions, the number of T-DNA right border fragments will equal the number of T-DNA left border fragments and this number will also represent the number of loci into which

the T-DNA has been integrated as well as the total number of FLAVR SAVR™ genes introduced into the tomato genome. This T-DNA border-type analysis has been commonly utilized to determine the number and organization of T-DNA regions inserted into transgenic plants (see, for example, Jorgenson *et al.* 1987).

Multiple copies of T-DNA regions inserted in tandem at a single plant DNA locus have been reported in the scientific literature (Jorgenson *et al.* 1987). For tandem insertions of T-DNA regions, both T-DNA:plant borders and T-DNA:T-DNA junctions will be detected using the Southern blot experimental design described above. A plant would be suspected of having multiple copies of tandemly arrayed T-DNA regions if the number of left T-DNA borders was not equal to the number of right T-DNA borders and/or the intensity of the hybridization signal was much stronger for some DNA fragments than for others. Junctions between adjacent T-DNA regions in tandem array can be verified since the lengths of junction DNA fragments produced by particular restriction enzymes can be predicted based on the known locations of their recognition sites in each of the adjacent T-DNA regions. Successful prediction of several junction fragment sizes, as judged by the results of several Southern blots prepared using several different restriction enzymes, serves as good evidence that the T-DNA region in a particular tomato plant is present in multiple, tandemly arrayed copies at a single locus.

Determining the total number of gene copies that have been inserted into an individual transformed plant can be accomplished by comparing the intensity of a Southern blot hybridization signal resulting from the introduced gene to the hybridization signal produced from a known amount DNA representing a known number of copies of the gene. Plasmid DNA used to produce the transformed plant can be used as a gene copy number standard. Also, because the FLAVR SAVR gene is complementary to the endogenous PG gene, a hybridization probe that recognizes the FLAVR SAVR gene will also recognize the endogenous PG gene, and the endogenous gene can serve as an internal standard for gene copy number. The endogenous PG gene is known to be present as one copy per haploid genome (Bird 1988). The PG gene has also been mapped with regard to restriction enzyme recognition sites (Bird 1988) and DNA fragments produced by restriction enzymes and representing the endogenous PG gene can be identified and distinguished, based on their molecular weight, from those representing the FLAVR SAVR gene in Southern blot experiments. The gene copy number type Southern blot experiments described below take advantage of the endogenous PG gene copy number standard. They have been designed so that possible signal differences, which can result from the differential transfer of DNA fragments of different sizes from the gel matrix to the solid membrane or differing extents of homology between the probe and the respective antisense PG or PG DNA fragment, have been minimized.

[redacted]

Southern blot experiments were also used to conduct analyses of the segregation of FLAVR SAVR™ genes and associated T-DNA border fragments among progeny of individual transgenic tomato plants. These analyses of progeny served two purposes. First, for those plants for which the T-DNA carrying the FLAVR SAVR gene was interpreted as being inserted into the tomato genome at a single locus, as is the case for all of the examples presented here, the inserted FLAVR SAVR gene should segregate in a simple Mendelian fashion. Of progeny resulting from self-fertilization of an original hemizygous plant, the ratio of those carrying the FLAVR SAVR gene to those without should be 3:1. This information should also agree with information on progeny segregation gained from kanamycin germination analysis. The presence or absence of the FLAVR SAVR gene was therefore tested in progeny using the Southern blot technique. This analysis also served as a test of the method to determine FLAVR SAVR gene copy number. The progeny which contain the FLAVR SAVR gene should comprise plants both homozygous and heterozygous for the FLAVR SAVR gene. These two classes of progeny should be distinguishable based on copy number of the inserted FLAVR SAVR gene as determined by Southern blot experiments described above. A second purpose of Southern blot experiments analyzing segregation among T_2 progeny is to verify the structure of the inserted T-DNA when multiple, tandemly arrayed T-DNA elements are present at one locus in the plant genome. If actually at one genetic locus, multiple hybridizing bands representing borders of the T-DNA region should co-segregate in subsequent generations. Analyzing the progeny of these plants serves as another confirmation of the proposed structure of inserted T-DNA carrying the FLAVR SAVR gene.

The Southern blot method can also be utilized to demonstrate physical linkage between the FLAVR SAVR gene and the *kan*^{r} selectable marker gene in transgenic tomatoes. These genes are physically linked within the T-DNA region of pCGN1436 and are therefore expected to be physically linked when inserted into the tomato genome. By treatment of genomic DNA with appropriate restriction enzymes, single DNA fragments that contain both the FLAVR SAVR gene and the *kan*^{r} gene should be produced which hybridize to probes representing either gene. The molecular weight of the fragments containing the two genes is predictable, based on the map of restriction enzyme recognition sites for pCGN1436, and probes representing both genes should therefore hybridize to a DNA fragment of that same molecular weight. Demonstrating the physical linkage between the FLAVR SAVR gene and the *kan*^{r} gene serves as an additional method to validate the analysis of T-DNA border fragments detailed below.

II. *kan*^{r} Gene Function.

A functionally active *kan*^{r} gene in genetically engineered tomato plants can be identified using either one of two methods. One method involves a germination assay in which seeds are sprouted in media containing the antibiotic kanamycin. In general, seedlings expressing the *kan*^{r} gene exhibit resistance in the form of long branching roots, while susceptible plants which do not contain the *kan*^{r} gene have seedlings with very short, unbranched roots with purple stems. A protocol for this procedure is described in Appendix D-3. The second method used to detect an active *kan*^{r} gene in genetically engineered plants utilizes an assay for APH(3')II activity and is generally carried out using leaf tissue samples from individual plants. This procedure is described by Radke *et al.* (1988).

If the T-DNA has been stably integrated into the plant genome, T_2 seed from a T_1 transgenic plant that has been self-fertilized would segregate for the inserted traits in simple Mendelian ratios. Thus, a segregation ratio of 3:1 kanamycin resistant to susceptible seedlings would indicate that the parent T_1 plant contained, at one genetically defined locus, at least one functionally active *kan*^{r} gene. As a consequence of the physical linkage of the *kan*^{r} gene and the FLAVR SAVR™ gene in transgenic plants (demonstrated below), a segregation ratio of 3:1 resistant to susceptible seedlings would also indicate the presence of the FLAVR SAVR gene in the parent plant at the same genetic locus.

III. Hybridization Probes and Map of pCGN1436.

DNA isolation and Southern analysis was performed as described in Section D.3.1.

DNA fragments utilized for hybridization probes were isolated from specific regions of plasmid DNA representing the pCGN1436 gene construct and are shown in Figure 1. The hybridization probes designed to identify the downstream end of the chimeric FLAVR SAVR gene (the *tml* 3' probe), consisted of the 1.2 kb SacI-PstI fragment. The hybridization probe designed to identify the chimeric *kan*^{r} gene coding region, the *kan*^{r} gene probe, consisted of the 1 kb EcoRI-EcoRI fragment shown in Figure 1. The hybridization probe designed to identify that portion of the chimeric *kan*^{r} gene coding region situated on the 5 kb SphI-SphI DNA fragment located within the T-DNA borders in pCGN1436, the sph *kan*^{r} probe, consisted of a 0.44 kb SphI-EcoRI fragment isolated from the *kan*^{r} gene EcoRI-EcoRI fragment just described (see Figure 1). The hybridization probe designed to identify both the introduced FLAVR SAVR gene and the endogenous PG gene, the PG

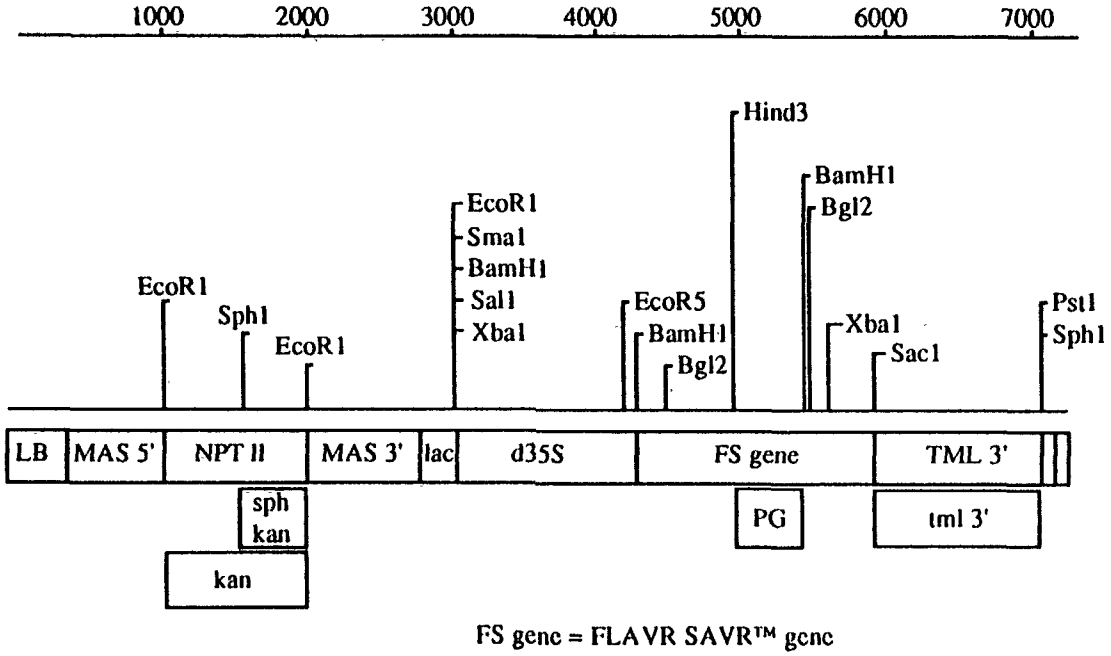


Figure 1. Map of T-DNA region of pCGN1436 with location of hybridization probes. Molecular weights given in bp.

probe, consisted of a 390 bp DNA fragment (see Figures 1 and 2). This fragment was isolated utilizing oligonucleotides
5'-ATCATAAGCTTGGAGATCTGGACAAGCTAG-3' and
5'-ATATCGAATTCAAAAGAGCTTCATCCTCTG-3' which were synthesized at Calgene and represent the boundaries of the desired fragment. These oligonucleotides were used as primers in a polymerase chain reaction (PCR) conducted with a DNA thermal cycler (Perkin Elmer Cetus), under standard reaction conditions suggested by the manufacturer, with template DNA containing the PG cDNA. The isolated PCR-produced fragment was then radiolabelled and used as a hybridization probe as described in Section D.3.1.

The membranes were hybridized with appropriate probes at 42°C, overnight. Non-specific hybridization was eliminated by washing the membranes in 0.1 X SSC, 0.1% SDS at 55° C for 1-2 hr. The membranes were then wrapped in plastic wrap and exposed to Kodak XAR film using an intensifying screen at minus 70°C unless otherwise indicated. Exposure times were generally 3 days. Membranes were stripped of radiolabeled probe by washing for 4 hr at 65°C in 250 ml of solution containing 1.25 ml 1M Tris pH 8.0, 0.5 ml 50X Denhardt's solution (Maniatis 1982), 0.2 ml 0.25 M EDTA and 0.125 g sodium pyrophosphate (Sigma).

IV. Analysis of the Number of FLAVR SAVR™ Genes Inserted into the Tomato Genome.

Based on the known location for sites at which the restriction endonucleases BamHI and BglII cut the FLAVR SAVR gene in pCGN1436 (Figure 1), a DNA fragment with a molecular weight of 967 bp and containing PG gene coding sequences was expected to be produced upon cutting genomic DNA from transgenic tomatoes with these two enzymes. A DNA fragment with a molecular weight of 1036 bp and containing endogenous PG gene coding sequences from the eighth and ninth exons as well as the intervening region between these exons (Figure 2 and Bird 1988) was expected to be produced upon cutting genomic DNA from either transgenic or control tomato plants with a mixture of these same two enzymes. The 390 bp PG gene probe (Figure 2) contains the eighth exon in its entirety and the 5' end of the ninth exon (to the BglII site) of the endogenous PG gene. The 390 bp PG gene probe is homologous to both the pCGN1436-derived 967 bp DNA fragment and the endogenous 1036 bp DNA fragment. A comparison of the hybridization signal intensity between the two DNA fragments when hybridized with this probe should therefore provide an estimate of the number of copies of each of the two genes present in the plant genome. The DNA fragment in this analysis derived from the endogenous PG gene represents two copies of that gene since the inbred lines used to produce the transgenic tomato cultivars are diploid. The endogenous PG gene is known to be present as one copy per haploid genome (Bird 1988).

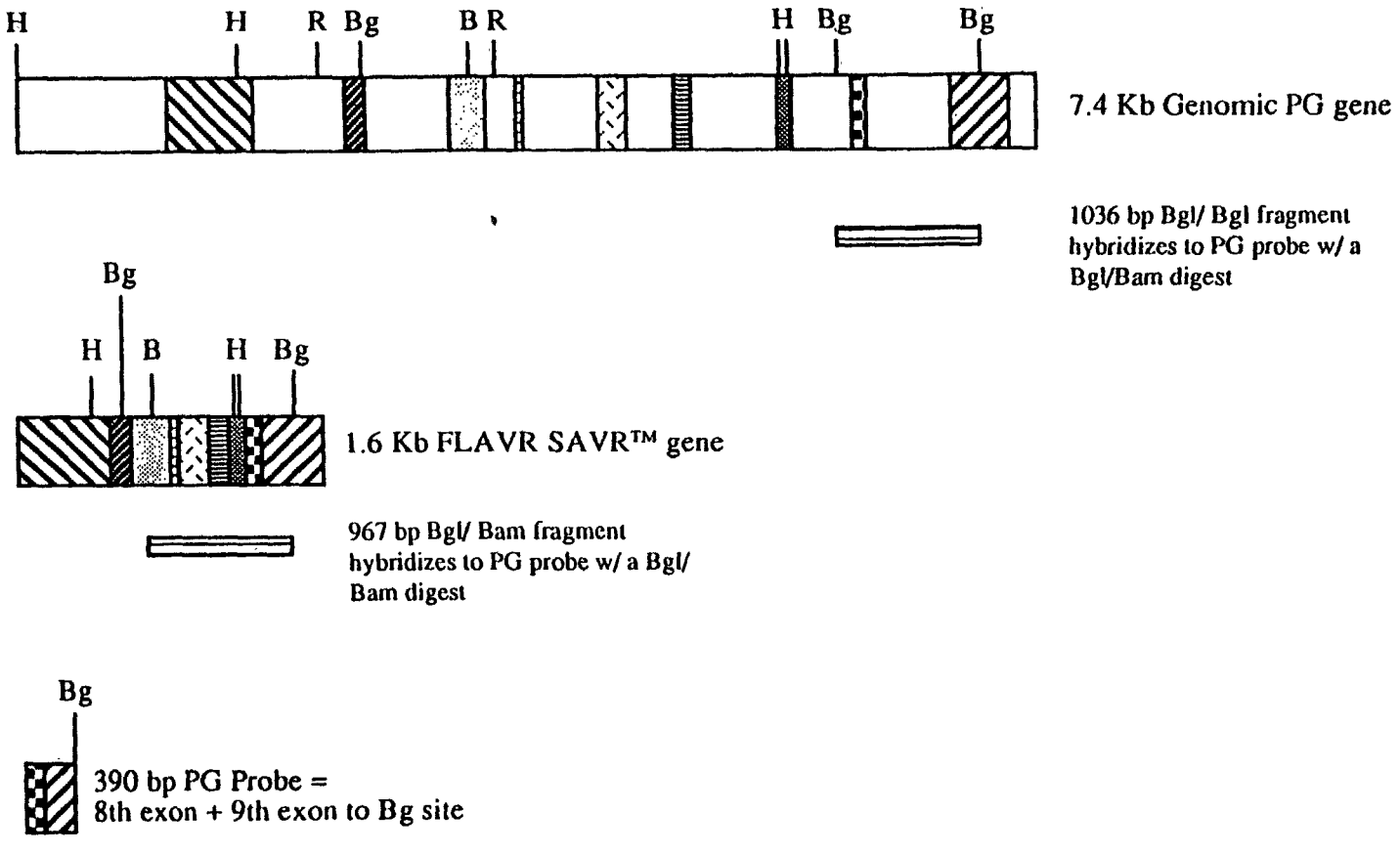


Figure 2. Experimental design of Southern blot experiment for determination of copy number of the FLAVR SAVR™ genes.

B = BamH1, Bgl = BglII, H = HindIII, R = EcoRI.

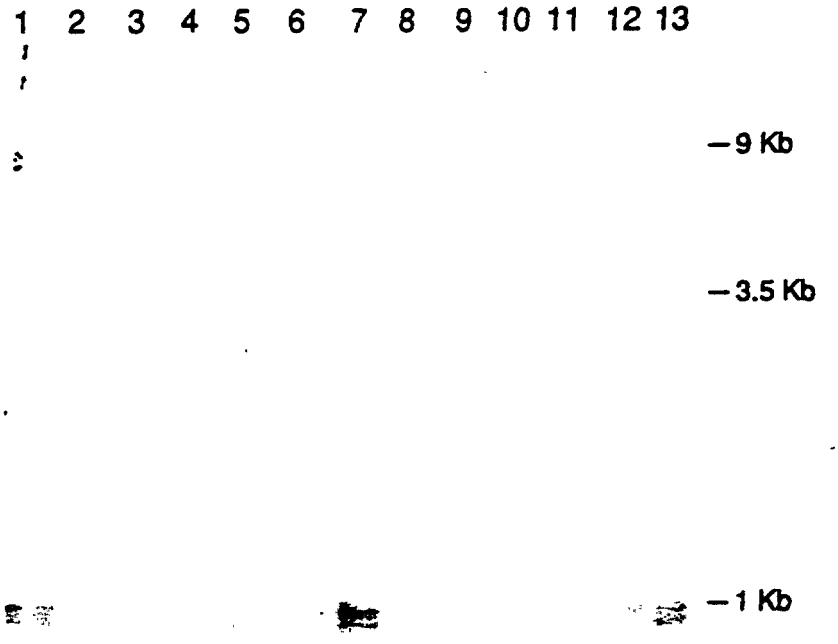
As shown in Figure 3, the expected 1036 bp and 967 bp DNA fragments are evident in all eight transgenic tomato lines examined in this experiment. The control plants produce only the 1036 bp DNA fragment representing two copies of the endogenous PG gene. A higher molecular weight DNA fragment also hybridizes with the PG probe in both transgenic and control DNA samples. This fragment probably represents another DNA sequence in the tomato genome that shares some homology with the PG gene. The 967 bp DNA fragment, representing the introduced FLAVR SAVR™ gene from pCGN1436, in transgenic tomato variety 501-1001 appears less intense, by approximately a factor of two, than the 1036 bp fragment representing the endogenous PG gene. The 967 bp antisense PG fragment in transgenic tomato variety 501-1035 appears more intense than the endogenous PG fragment. The antisense PG DNA fragment in each of the other six transgenic tomato varieties examined in this experiment is approximately equal in hybridization signal intensity to the endogenous PG fragment in each individual. An additional DNA fragment of high molecular weight (approximately 9 kb) also hybridizes to the PG probe in the DNA sample isolated from transgenic line 22B-215. Initially, this band appeared to have resulted from partial digestion of this DNA sample. No other unexplained hybridization bands were observed for this transformant, or for any of the other seven lines of FLAVR SAVR tomatoes examined (see below) using this or any of the other combination of restriction and hybridization probes used for this analysis. Further analyses would be required to determine whether the hybridization band observed for transformant in the experiment shown in Figure 3. 22B-215 represents an additional FLAVR SAVR gene(s) in the 22B-215 genome or an artifact. These results indicate that only one copy of the FLAVR SAVR gene has been inserted into the genome of the 501-1001 plant, two copies have been inserted into the genomes of plants 502-2021, 7B-92, 28B 419, -425 and -498, and more than two copies have been inserted into the genome of plant 501-1035.

V. Physical Linkage of FLAVR SAVR Gene and *kan*^{r} Gene.

The following experiments were conducted to determine whether the *kan*^{r} gene had remained physically linked to the FLAVR SAVR gene. As shown in Figure 4, the restriction endonuclease SphI cuts pCGN1436 DNA generating a DNA fragment of 5 kb molecular weight and containing the 3' portion of the *kan*^{r} gene and the entire FLAVR SAVR gene. As can be seen in Figure 5, hybridization of the PG gene probe to the expected 5 kb fragment of DNA is observed only in the lanes containing DNA isolated from the transformed plants. No hybridization to a 5 kb fragment is observed in the lanes containing DNA isolated from the nontransformed, control plants. Hybridization to higher molecular weight fragments is also observed in all the DNA samples with this PG probe and these fragments are assumed to represent the endogenous PG gene. The additional high molecular weight band observed in 22B-215 was later shown to be the result of partial digestion.

Figure 3. Southern blot of DNA from T_1 tomato transformants digested with Bam HI and BglII and hybridized with PG probe. Lane assignments are as follows:

(1) 502-2021, (2) 502 control, (3) 501 control, (4) 501-1001, (5) 501-1035, (6) 22B control, (7) 22B-215, (8) 28B control, (9) 28B-419, (10) 28B-425, (11) 28B-498, (12) 7B control, (13) 7B-92.



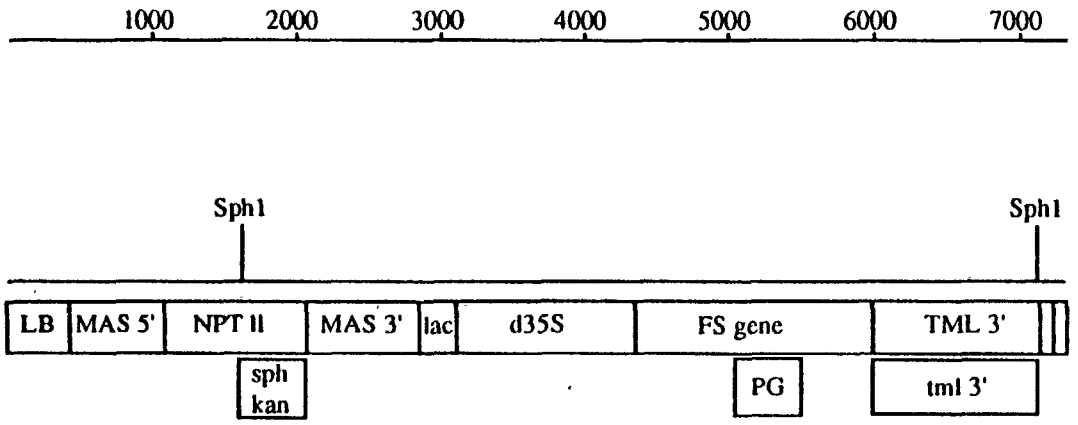
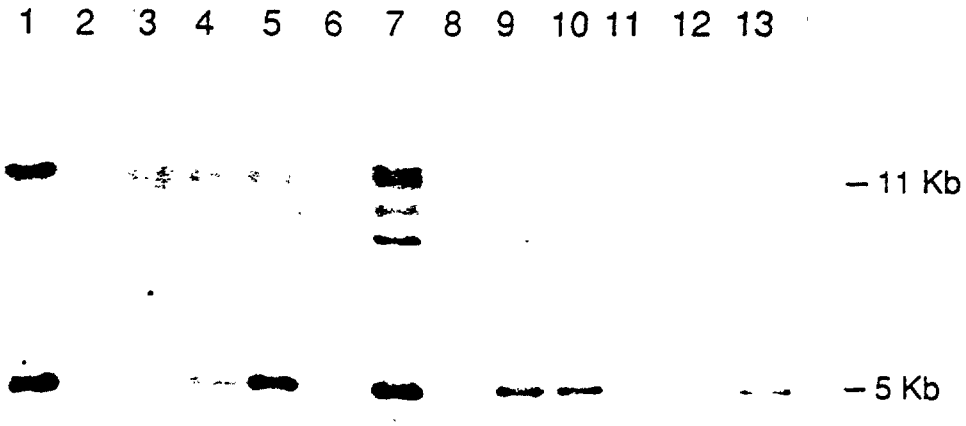


Figure 4. Probes and restriction enzymes used to show linkage of *kan*^{r} and FLAVR SAVR™ genes. Molecular weights given in bp.

Figure 5. Southern blot of DNA from T_1 tomato transformants digested with SphI and hybridized with PG probe. Lane assignments as in legend to Figure 3.



The PG gene hybridization probe was then removed from the Southern blot shown in Figure 5. and the stripped blot was used to expose Kodak XAR film. After three days the film was developed and no evidence of the PG gene hybridization signal found. The blot was then rehybridized with the 440 bp sph *kan*^{r} gene probe (Figure 4) to identify *kan*^{r} gene coding sequences on the 5 kb fragment. As can be seen in Figure 6, hybridization of a DNA fragment of the expected 5 kb molecular weight is observed in the lanes containing DNA isolated from the transformed plants. Again, no hybridization is observed in the lanes containing DNA isolated from the nontransformed, control plants.

The *tml* 3' probe was also used to identify the 5 kb SphI DNA fragment in the eight transgenic tomato lines. The sph *kan*^{r} gene hybridization probe was removed from the Southern blot shown in Figure 6 and the blot examined for any residual *kan*^{r} gene probe as described above for the PG probe. The blot was then rehybridized with the *tml* 3' probe which represents the 3' end of the FLAVR SAVR™ gene (see Figure 4). As can be seen in Figure 7, hybridization to the expected 5 kb fragment of DNA using this probe is also observed only in the lanes containing DNA isolated from the transformed plants. No hybridization to a 5 kb fragment, or any other DNA, is observed in the lanes containing DNA isolated from the nontransformed, control plants. An additional DNA fragment is observed in the lane containing DNA isolated from plant 501-1001. Because this fragment is not the molecular weight expected based on the DNA sequence of pCGN1436 (see Figure 4), it is assumed that the DNA sequence adjacent to this fragment has been altered as compared to the pCGN1436. These results are interpreted as indicating that in addition to a complete copy of the T-DNA from pCGN1436, an incomplete copy of the region has also been inserted into the genome of 501-1001, and data demonstrating the linkage of this fragment will be discussed below.

In summary, the data presented above demonstrate that probes representing the *kan*^{r} gene and the FLAVR SAVR gene (both PG gene "coding" sequences and the *tml* 3' untranslated region in the chimeric antisense PG gene construction) all hybridize to a predicted 5 kb fragment produced by Sph digestion of DNA isolated from plants transformed with pCGN1436. These results indicate that the *kan*^{r} and the FLAVR SAVR genes have remained physically linked in the transformed plants described here.

VI. Analysis of the Number of Sites in the Tomato Genome into which FLAVR SAVR Genes Have Been Inserted.

For plants containing more than one copy of the FLAVR SAVR gene, the data described above give no indication of the number of T-DNA insertions in each plant's genome. To determine the number of insertion sites of FLAVR SAVR genes, experiments were done to find the number of borders between

Figure 6. Southern blot of DNA from T_1 transformants digested with SphI and hybridized with sph *kan*^{r} probe. Lane assignments as in legend to Figure 3.

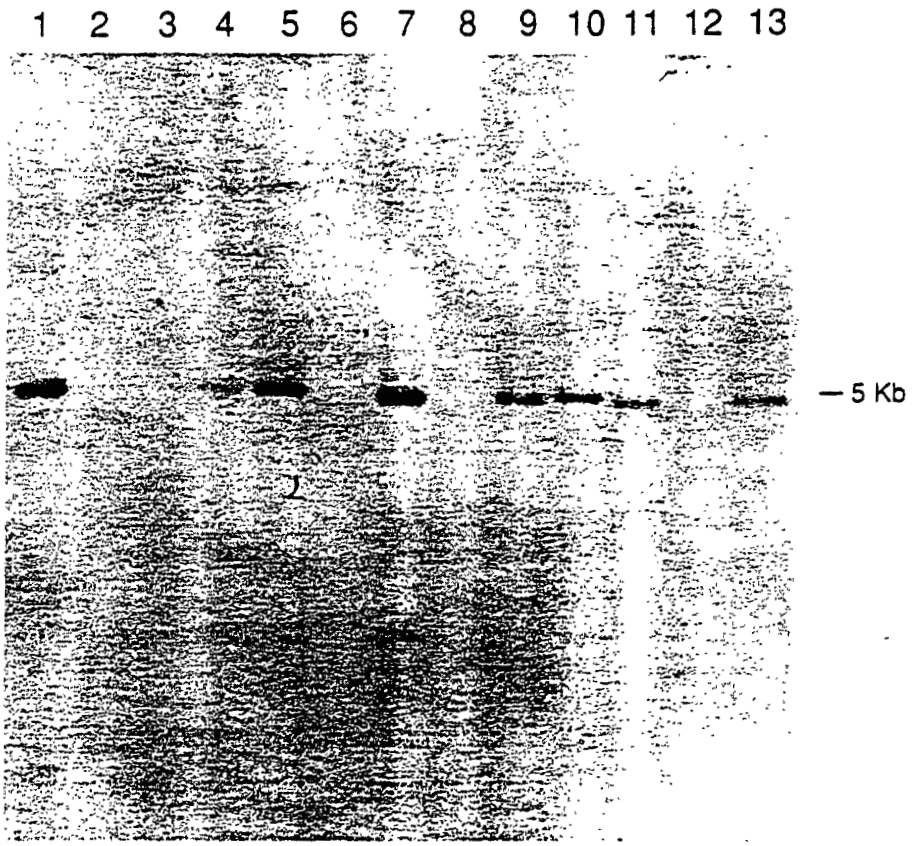
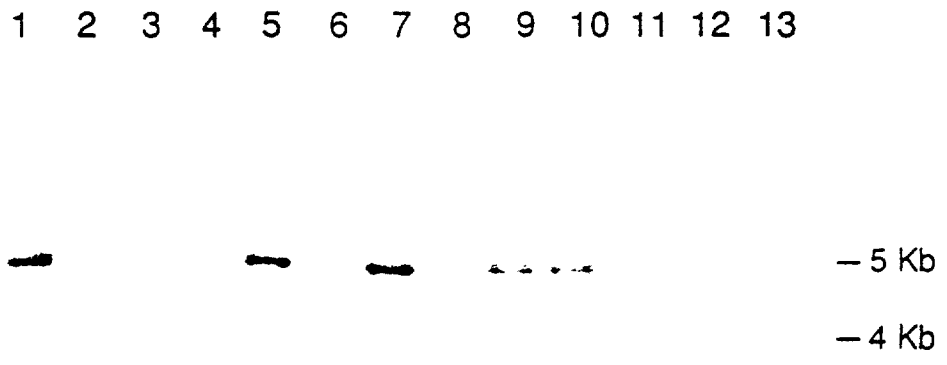


Figure 7. Southern blot of DNA from T_1 transformants digested with SphI and hybridized with *tml* 3' probe. Lane assignments as in legend to Figure 3.



the T-DNA regions containing the FLAVR SAVR™ gene, and plant DNA. As shown in Figure 8, the *kan*^{r} and *tml* 3' probes were used to identify the left and right borders of the inserted T-DNA, respectively. This approach is valid since physical linkage of the regions represented by these probes was demonstrated in Section V above for the eight transformants examined.

To detect borders involving the end of the T-DNA containing the right border repeats and the FLAVR SAVR gene, genomic DNA from the transgenic tomato plants was cut with the restriction endonucleases BamHI and BglII. Based on the known location for sites at which these restriction endonucleases cut pCGN1436 DNA, a DNA fragment of at least 1.9 kb was expected to hybridize to the *tml* 3' probe (Figure 8). The actual length of the hybridizing DNA fragment depends on the location of a BamHI or BglII recognition site in the plant genome at an unknown distance from the site at which the T-DNA was inserted. Therefore, the exact size of the resulting fragment cannot be predicted. As can be seen in Figure 9, hybridization of a single DNA fragment larger than 1.9 kb is observed in DNA isolated from each of the transgenic plants examined. No hybridization is observed in the lanes containing DNA isolated from the nontransformed, control plants.

To examine borders involving the end of the T-DNA containing the left border repeats and the *kan*^{r} gene, the Southern blot shown in Figure 9 was stripped of its previous hybridization probe and rehybridized with the *kan*^{r} gene probe. Based on the known location of BglII and BamHI sites in pCGN1436, a DNA fragment of at least 2 kb was expected to hybridize with this probe (Figure 8). As with the length of right T-DNA border fragments, the length of the left border fragments cannot be predicted. As shown in Figure 10, hybridization of one T-DNA left border DNA fragment larger than 3 kb is observed with DNA isolated from transgenic lines 501-1001 and 28B-425. In the case of 28B-425, the apparent single band is likely to be a doublet because digestion of 28B-425 DNA with XbaI and hybridization with the *kan*^{r} gene probe resulted in two left border fragments (see Figure 11). The other six transgenic lines show two hybridizing left border fragments. No hybridization is observed in the lanes containing DNA isolated from the nontransformed, control plants.

Transgenic tobacco plants resulting from *Agrobacterium* transformation and regeneration have been shown to have multiple adjacent T-DNA copies in head to head orientation (Jorgensen *et al.* 1987). Such a T-DNA configuration is consistent with the data depicted in Figures 9 and 10. The molecular weight of the fragment from the right border analysis (approximately 4 kb, see Figure 9) is the same in all seven of the transgenic lines in which multiple copies of the FLAVR SAVR gene had been observed in the experiment shown in Figure 3. It is highly unlikely that a BamHI or BglII recognition site would occur in plant DNA at the same distance from the right border of the T-DNA region in each of these independently derived plants. In addition, 4 kb is the [redacted]

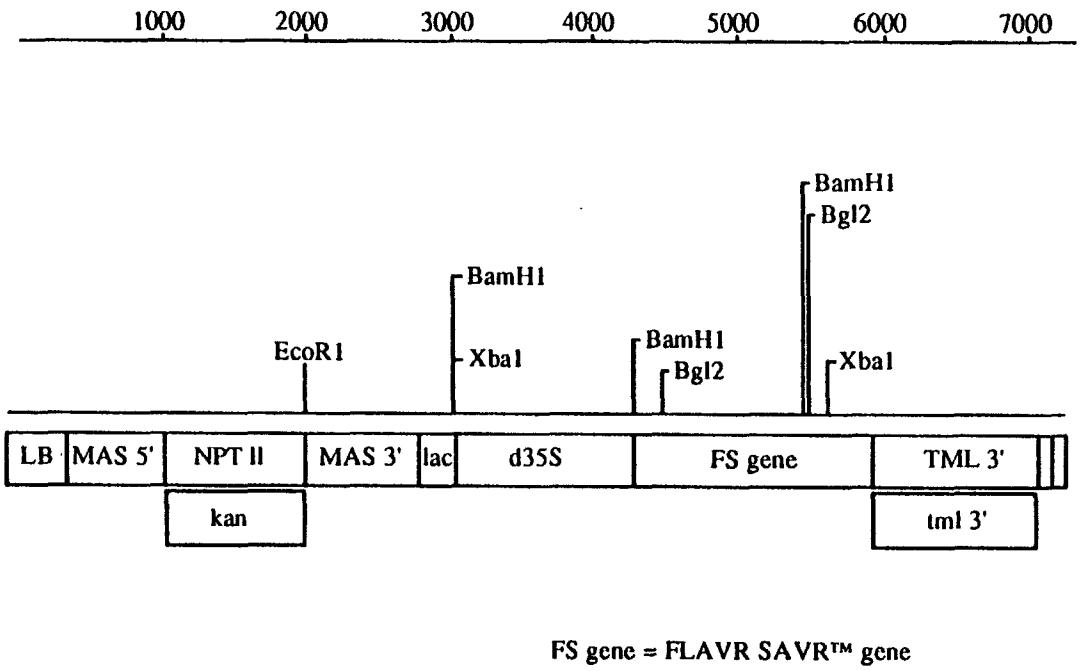


Figure 8. Probes and restriction enzymes used to determine number of border fragments. Molecular weights given in bp.

Figure 9. Southern blot of DNA from T_1 transformants digested with BamHI and BglII and hybridized with *tml* 3' probe. Lane assignments as in legend to Figure 3.

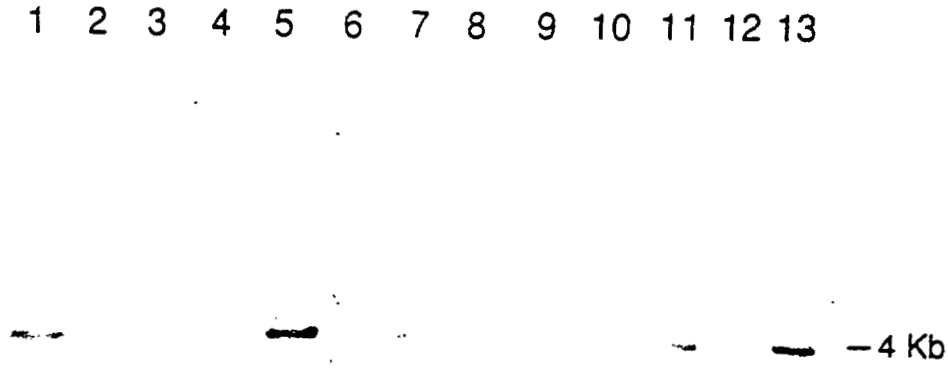


Figure 10. Southern blot of DNA from T1 transformants digested with BamHI and BglII and hybridized with *kan*^{r} probe. Lane assignments as in legend to Figure 3.

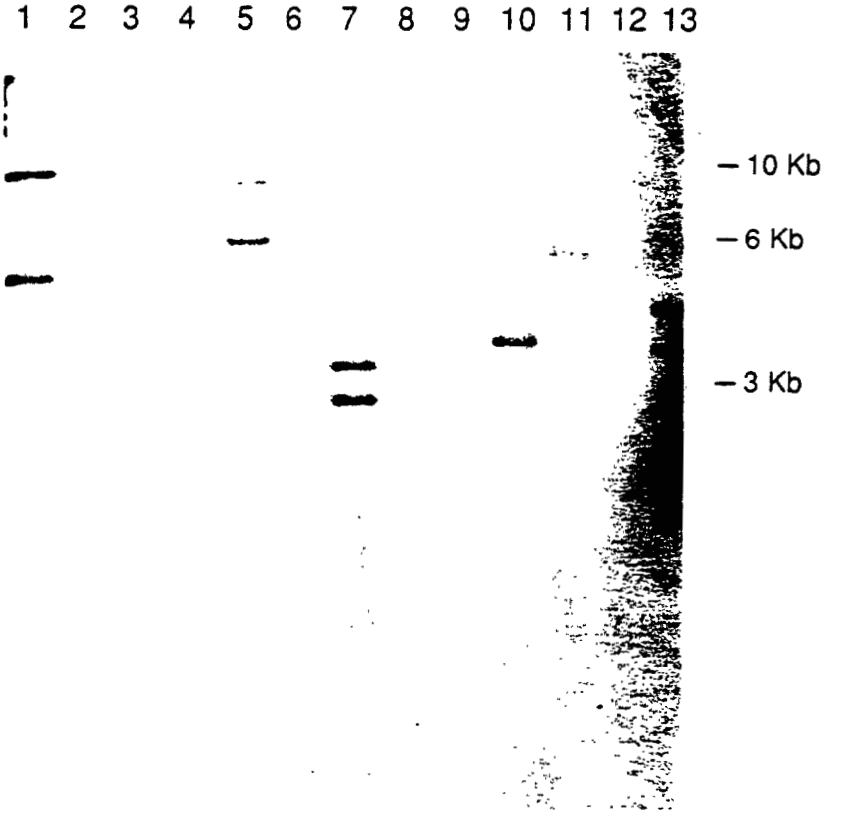


Figure 11. Southern blot of 28B-425 DNA digested with XbaI and hybridized with the *kan*^{r} probe. Lane (1) 28B control, (2) 28B-425.

[redacted]

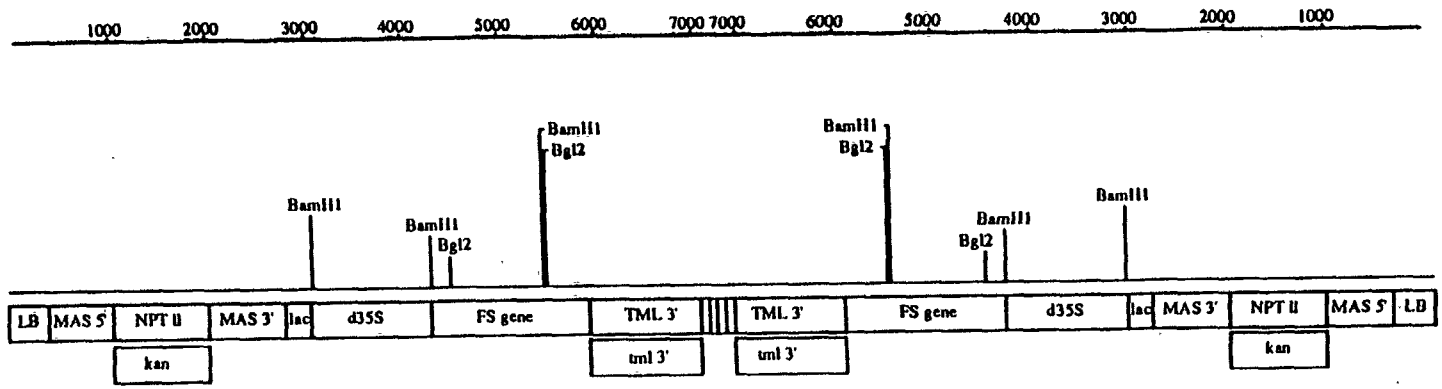
molecular weight expected of a fragment serving as a junction between two T-DNA regions adjacent at their right borders, as shown in Figure 12. Assuming the T-DNA regions in these plants had been inserted in such a head to head configuration, with the "head" being the T-DNA right border, two left border fragments should be detected using the *kan*^{r} gene probe. Based on the data shown in Figures 10 and 11, two left border fragments were observed using this probe to hybridize to DNA isolated from all seven lines which potentially have their T-DNA regions inserted in this configuration.

Further Southern analysis was conducted to detect specific junction fragments predicted between adjacent T-DNA regions if in a head to head orientation. As can be seen in Figure 13, hybridization, using the *tml* 3' gene probe, to one DNA fragment of approximately 8 kb is predicted for EcoRI-cut DNA from plants suspected of having adjacent T-DNA regions inserted into their genomes at one genetic locus. As shown in Figure 14, this is the case of all plants except 501-1001 and 501-1035. Thus, the structure of the T-DNA inserts for transgenic lines 502-2021, 7B-92, 22B-215, and 28B-419, -425, and -498 appears to be as shown in Figure 13.

Plant 501-1001 was previously shown to have two copies of the *tml* 3' sequence (Figure 7), but only a single fragment that hybridized to the *tml* 3' probe was observed in this EcoRI digest (Figure 10) or in an XbaI digest (data not shown). These observations, in addition to the fact that neither the PG probe (Figure 5) nor the sph *kan*^{r} probe (Figure 6) hybridized to the additional Sph DNA fragment in plant 501-1001 observed hybridizing to the *tml* 3' probe in lane 4 of Figure 7 suggest a structure for transgenic line 501-1001 as shown in Figure 15. No hybridization is observed in the lanes containing DNA isolated from the nontransformed control plants.

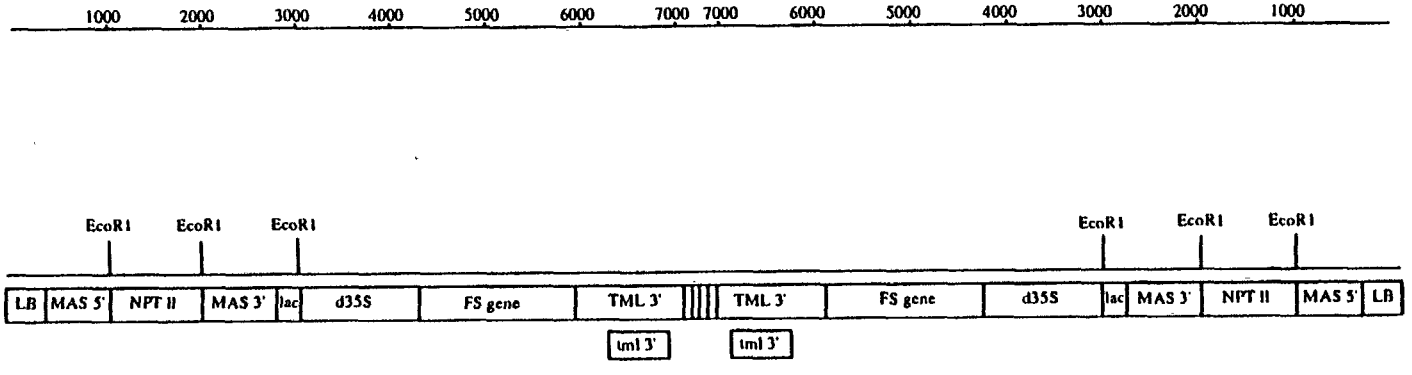
In addition to the 8 kb EcoRI fragment discussed above, transgenic line 501-1035 also contained a 4.5 kb EcoRI fragment that hybridized to the *tml* 3' probe (lane 5, Figure 14). This result is consistent with there being a third copy of the T-DNA region at one genetic locus if it is assumed that the three copies of the T-DNA are arranged in a head to head then tail to tail configuration, as shown in Figure 16. This structure is also consistent with the observation that more than two copies of the FLAVR SAVR™ gene had been inserted into the genome of this plant (see Figure 3).

An additional Southern blot experiment with DNA isolated from transgenic line 501-1035 was conducted to show that the three T-DNA copies in 501-1035 were linked as in Figure 16. Samples of 501-1035 DNA were cut with various restriction enzymes each of which should produce a DNA fragment of a predictable molecular weight representing a junction between adjacent T-DNA region left borders, and a DNA fragment of an unpredictable molecular weight representing a border between the left end of the T-DNA and the adjacent plant DNA. A junction fragment between adjacent left T-DNA



FS gene = FLAVR SAVR™ gene

Figure 12. Structure of T-DNA inserts predicted for transgenic lines 502-2021, 22B-215, 28B-419, 28B-425, 28B-498 and 7B-92 from Southern blots shown in Figures 10 and 11.

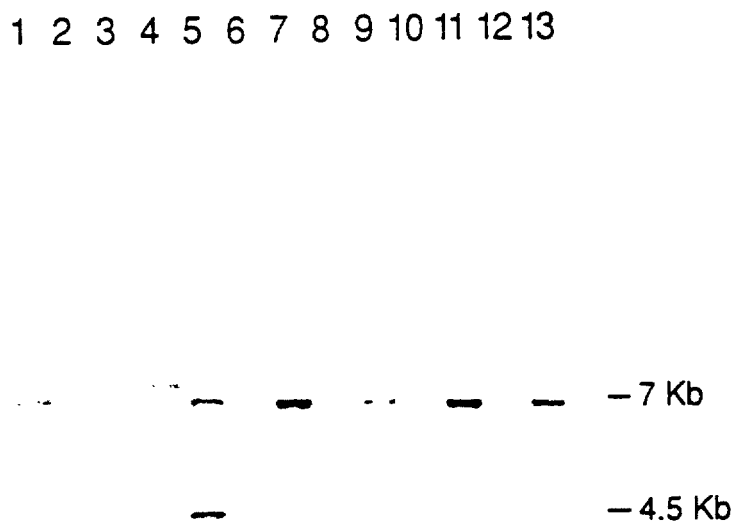


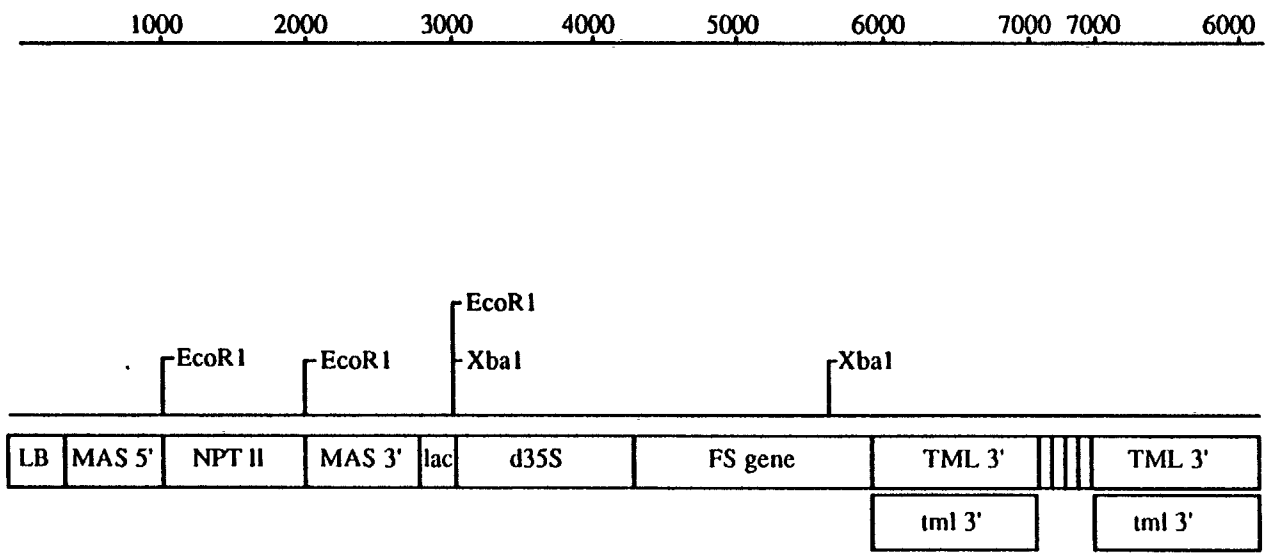
FS gene = FLAVR SAVR™ gene

Figure 13. Probe and restriction enzyme used to demonstrate junction fragments between adjacent T-DNAs. Molecular weights given in bp.

Figure 14. Southern blot of DNA from T_1 transformants digested with EcoRI and hybridized with *tml* 3' probe. Lane assignments are as follows:

(1) 502-2021, (2) 502 control, (3) 501 control, (4) 501-1001, (5) 501-1035, (6) 7B control, (7) 7B-92, (8) 22B control, (9) 22B-215, (10) 28B control, (11) 28B - 419, (12) 28B - 425, (13) 28B - 498.





FS gene = FLAVR SAVR™ gene

Figure 15. Proposed structure of T-DNA insert in transgenic line 501-1001.

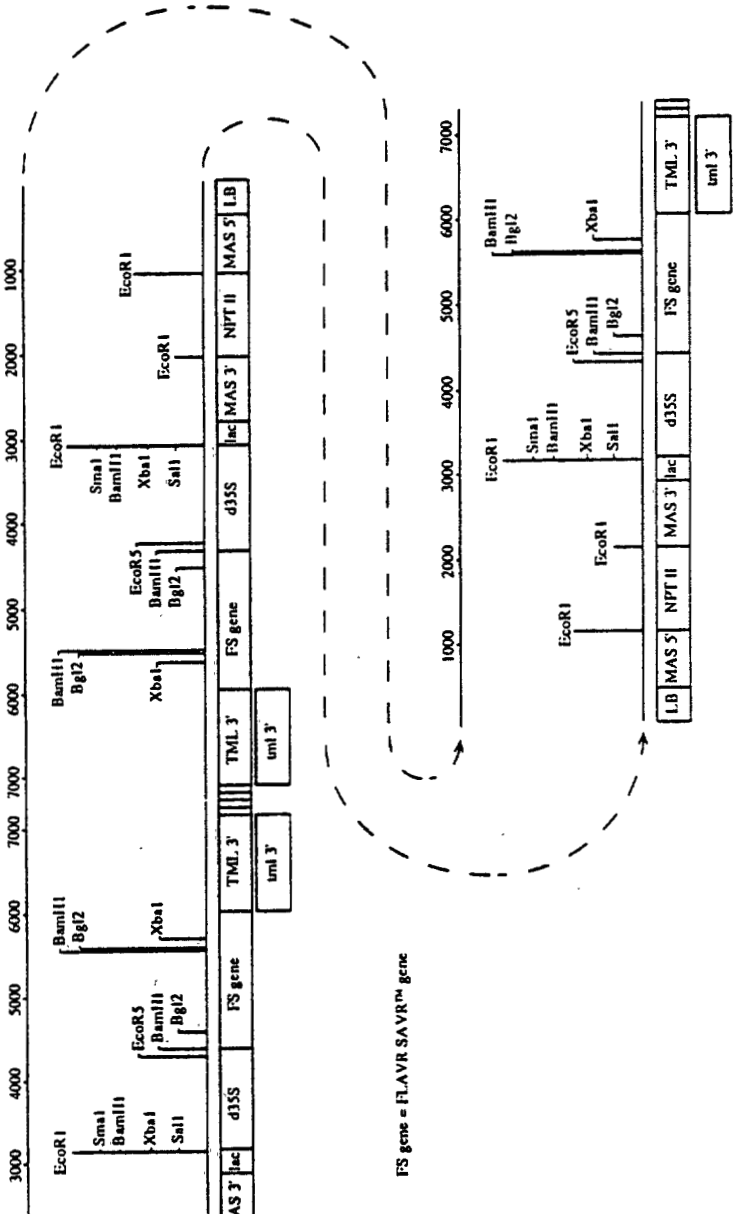


Figure 16. Probes and restriction enzymes used to test proposed structure for T-DNA insert in transgenic line 501-1035. Molecular weights given in bp.

borders of approximately 6 kb molecular weight was expected if SmaI, BamHI, XbaI or SalI were used to cut genomic DNA from plant 501-1035. The molecular weight of a junction fragment between adjacent left T-DNA borders expected when plant 501-1035 DNA was cut with EcoRV would be approximately 8.4 kb. These fragments, as well as an additional border fragment in each case, would contain the *kan*^{r} gene and should therefore be detected using a *kan*^{r} gene probe. As shown in the Southern blot experiment depicted in Figure 17, each of the predicted junction fragments between adjacent left T-DNA borders are evident in digests of digests of DNA isolated from transgenic line 501-1035. An additional fragment of an unpredicted molecular weight and interpreted as representing a border fragment in each case is also observed in each experimental lane. These data are consistent with the structure for inserted T-DNA regions for 501-1035 as shown in Figure 16.

VII. Analysis of T_2 Progeny of the Transgenic Lines.

Southern analysis of the T_2 progeny resulting from self-fertilization of the eight transgenic plants described above was done to provide an additional demonstration that the FLAVR SAVR™ and *kan*^{r} genes are inserted at a single locus. For example, if the two DNA fragments that hybridized with the *kan*^{r} gene probe in the experiment shown in Figure 10 represent border fragments (or, in the case of line 501-1035, one border and one junction fragment) derived from an inverted repeat configuration of T-DNA regions at a single genetic locus, then those DNA fragments should co-segregate among the progeny of those plants. That is, in the three out of four progeny plants which are kanamycin-resistant, the same two fragments which hybridize to the *kan*^{r} probe should be evident. If, on the other hand, the two DNA fragments hybridizing to the *kan*^{r} gene probe represent the border fragments of multiple T-DNA insertions at different loci in the tomato genome, then the fragments would be expected to segregate independently in the T_2 progeny, assuming sufficient genetic distance between the two loci. In the latter case, some of the kanamycin-resistant progeny plants examined would be expected to have only one or the other of the fragments hybridizing to the *kan*^{r} gene probe.

To examine the segregation pattern of DNA fragments hybridizing with the *kan*^{r} gene probe, Southern blot experiments were conducted on DNA isolated from T_2 progeny of the eight transgenic lines and cut with a mixture BamHI and BglII enzymes. As an example, results of this analysis for progeny of line 28B-419 (Figure 18) and 501-1035 (Figure 19) are shown. Eleven to 12 T_2 progeny of each plant were analyzed. Progeny of the T_1 plants that had multiple hybridizing T-DNA bands showed the same hybridizing bands in (roughly) 3/4 of the T_2 progeny plants. In no case did a T_2 plant contain only one of the DNA fragments which would have demonstrated a lack of linkage

Figure 17. Southern blot of 501-1035 DNA digested with the following restriction enzymes: Lane (1) BamHI, (2) EcoRV, (3) SalI, (4) SmaI, (5) XbaI and hybridized with *kan*^{r} probe.

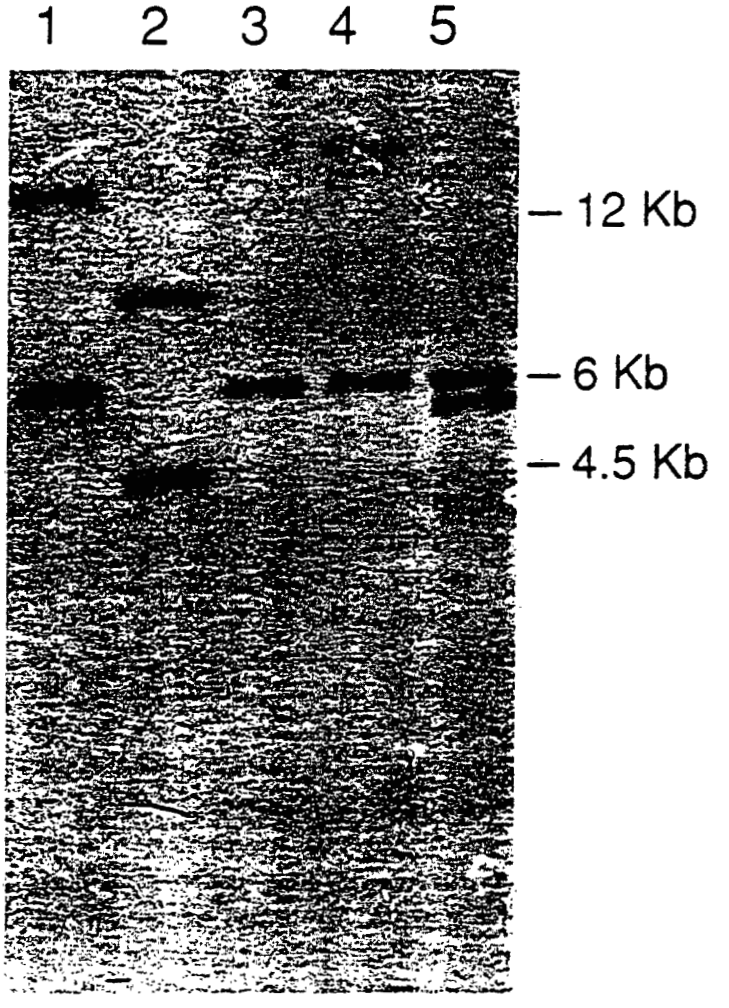


Figure 18. Southern blot of DNA from T_2 progeny of transgenic line 28B - 419 digested with BamHI and BglII and hybridized with the *kan*^{r} probe. DNA from the original T_1 28B-419 plant is shown in the far left lane. Individual T_2 progeny are designated by number.

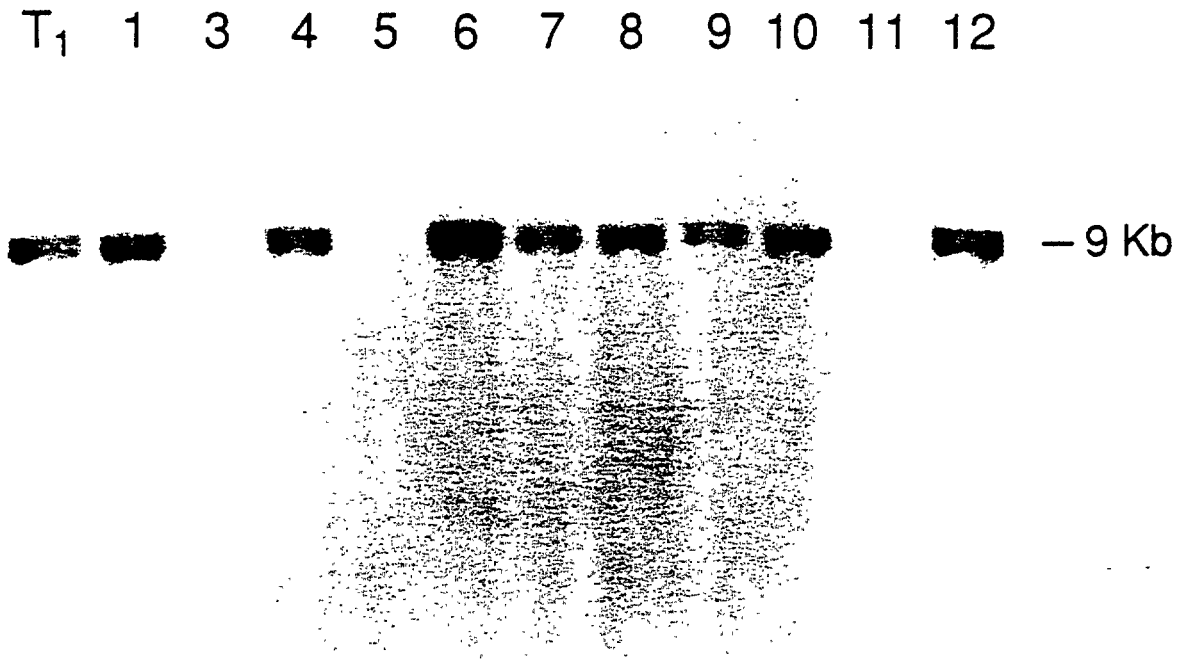


Figure 19. Southern blot of DNA from T_2 progeny of transgenic line 501-1035 digested with BamHI and BglII and hybridized with *kan*^{r} probe. DNA from the original T_1 501-1035 plant is shown in the far right lane. Individual T_2 progeny are designated by number.

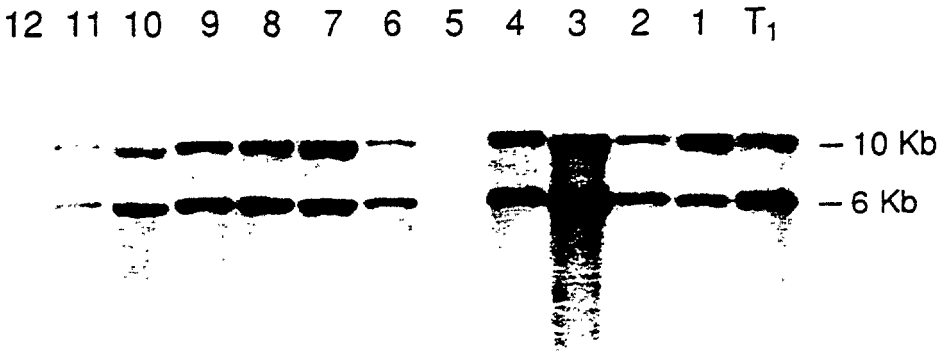


Table 1. Analysis of T_2 Progeny of Parental Plants Containing the FLAVR SAVR™ Gene.

P = Parental pattern (i.e., hemizygous)
N = No hybridization of expected FLAVR SAVR gene (i.e., homozygous null)
H = Antisense PG fragment twice as intense as in parent plant DNA (homozygous positive for FLAVR SAVR gene)
* = Ratio of resistant to susceptible T_3 progeny seedlings (Appendix D-3); the incidence of a small number of individuals designated resistant among progeny of plants determined to be null both in terms of APH(3')II activity and Southern analysis is likely due to the subjectivity of the germination assay.
n.d. = Not determined.
IP = In progress.

Progeny	APH(3')II Activity	Southern Results (P, N, H)	Germination Assay of T_3 seed *
1001-1	+	P	6/1
1001-3	+	P	16/8
1001-4	-	N	0/15
1001-5	+	P	15/4
1001-6	+	H	13/0
1001-7	-	N	0/18
1001-8	+	P	4/9
1001-9	+	P	14/1
1001-10	+	P	9/15
1001-11	+	H	27/0
1001-12	+	H	23/0
425-1	-	N	0/24
425-2	+	H	34/0
425-3	-	N	3/21
425-4	+	P	20/6
425-5	+	P	33/4
425-6	-	N	3/21
425-7	-	N	n.d.
425-8	-	N	0/12
425-9	-	N	0/12
425-10	+	P	8/4
425-11	+	P	19/5
425-12	+	P	23/6
419-1	+	P	n.d.

[redacted]

419-3	-	N	0/22
419-4	+	P	27/9
419-5	+	P	41/5
419-6	+	H	32/0
419-7	+	P	26/4
419-8	+	P	24/8
419-10	+	P	n.d.
419-11	+	P	n.d.
419-12	-	N	0/19
419-14	+	H	22/0
1035-1	+	P	4/1
1035-2	+	P	11/3
1035-3	+	H	25/0
1035-4	+	H	4/0
1035-5	-	N	0/18
1035-6	+	P	17/4
1035-7	+	P	2/1
1035-8	+	P	1/1
1035-9	+	P	17/1
1035-1F	+	P	n.d.
1035-2F	+	P	n.d.
1035-3F	-	N	n.d.
2021-1	-	N	0/29
2021-2	+	H	30/0
2021-3	-	N	0/31
2021-4	-	N	0/31
2021-5	+	H	41/0
2021-6	+	n.d.	7/0
2021-8	-	N	0/13
2021-9	-	N	0/16
2021-10	+	P	17/5
2021-11	+	P	17/3
2021-12	+	H	23/0
92-1	+	H	27/0
92-2	+	H	n.d.
92-3	+	n.d.	14/2
92-4	+	P	n.d.
92-6	+	H	16/0
92-7	+	P	n.d.
92-10	+	P	29/4
92-12	+	H	29/1
92-14	+	P	12/1
92-15	+	P	20/4

[redacted]

92-16	+	H	n.d.
92-20	+	P	34/6
498-1	+	H	n.d.
498-2	+	P	19/2
498-3	-	N	IP
498-4	+	P	36/5
498-5	+	P	IP
498-6	+	P	IP
498-7	-	N	n.d.
498-8	+	H	IP
498-9	-	N	n.d.
498-10	+	P	28/5
498-11	+	P	n.d.
498-12	-	N	4/26
215-1	+	P	48/13
215-6	+	H	40/0
215-7	-	N	n.d.
215-10	+	P	41/14
215-11	+	P	n.d.
215-12	+	P	27/6
215-14	+	P	n.d.
215-15	+	P	28/11
215-16	+	P	14/3
215-18	-	N	n.d.
215-19	-	N	0/32

and indicated multiple insertion sites. Progeny lacking fragments homologous to the *kan*^{r} probe also did not express APH(3')II activity, as expected (Table 1).

To examine the number of copies of the FLAVR SAVR™ gene in the progeny of the eight transgenic lines, the Southern blots described above were stripped of the *kan*^{r} gene probe and rehybridized with the PG gene probe. If each original parent carried the T-DNA regions with the copies of the FLAVR SAVR gene and the active *kan*^{r} genes at only one genetic locus, then three genetic classes were expected to be identified in these experiments. Approximately 25% of the progeny from a particular parent were expected to have the hybridization pattern observed for a nontransformed control plant. These progeny would represent the homozygous null genetic class; the FLAVR SAVR and the *kan*^{r} genes would be eliminated from these plants as a result of genetic segregation of the chromosome carrying the T-DNA regions during meiosis in the parent plant. Approximately 50% of the progeny were expected to have the same hybridization pattern as had been observed for the

T_1 parent plant. These progeny would represent the heterozygous (or hemizygous) genetic class. Only one chromosome of a homologous pair would carry the FLAVR SAVR™ and *kan*^{r} genes in these plants. Another 25% of the progeny were expected to carry the FLAVR SAVR and *kan*^{r} genes on both the homologues of a chromosome pair and thereby have a hybridization signal with the PG probe that was twice as intense as was observed in the parent plant. Representative results of the analysis of the progeny of transgenic lines 501-1001, and 28B-425 are shown in Figures 20 and 21.

In two of the eleven progeny plants examined in the 501-1001 experiment, 1001-4 and 1001-7, only the 1036 bp BglII-BglII DNA fragment representing the endogenous PG gene is observed hybridizing with the PG gene probe (Figure 20). Leaves from progeny plants 1001-4 and 1001-7 were also negative for APH(3')II activity (Table 1). These results demonstrate that the FLAVR SAVR and *kan*^{r} genes were not present in these progeny. In progeny plants 1001-6, 1001-11 and 1001-12 the 967 bp antisense PG gene fragment and the 1036 bp endogenous PG gene fragment hybridized to the PG gene probe with equal intensity indicating that two copies of the FLAVR SAVR gene are present in these plants. Hybridization of the PG gene probe to the 967 bp antisense PG gene fragment in the other six progeny plants examined resulted in a hybridization signal half the intensity of that observed for the 1036 bp endogenous PG gene fragment, as was the case for the T_1 parent 501-1001 plant. These results indicate that these plants contain only one copy of the FLAVR SAVR gene.

As can be seen for the progeny of transgenic line 28B-425 (Figure 21), in six of the twelve individuals analyzed only the 1036 bp BglII-BglII DNA fragment representing the endogenous PG gene is observed hybridizing with the PG gene probe. Leaves from these six progeny were also negative for APH(3')II activity (Table 1), indicating that the FLAVR SAVR and *kan*^{r} genes are not present in these progeny. In another five individual progeny the 967 bp antisense PG gene fragment and the 1036 bp endogenous PG gene fragment hybridized to the PG gene probe with equal intensity. These plants thus have two copies of the FLAVR SAVR gene, as was the case for the parent T_1 plant. In one individual, 425-2, the hybridization signal of the 967 bp FLAVR SAVR gene fragment was approximately twice as intense as that of the DNA fragment representing the endogenous PG gene. Progeny plant 425-2 was homozygous in terms of the *kan*^{r} gene as judged by the results of a kanamycin germination assay with seed from this individual (see Table 1).

The results of similar experiments determining the number of copies of the FLAVR SAVR gene in progeny of the other transgenic lines under investigation are presented in Table 1. In summary, the three genetic classes expected for progeny resulting from self-fertilization of each T_1 transgenic

Figure 20. Southern blot of DNA from T_2 progeny of transgenic line 501-1001 digested with BamHI and BglII and hybridized with PG probe. DNA from the original T_1 501-1001 plant is shown in the far right lane. Individual T_2 progeny are designated by number.

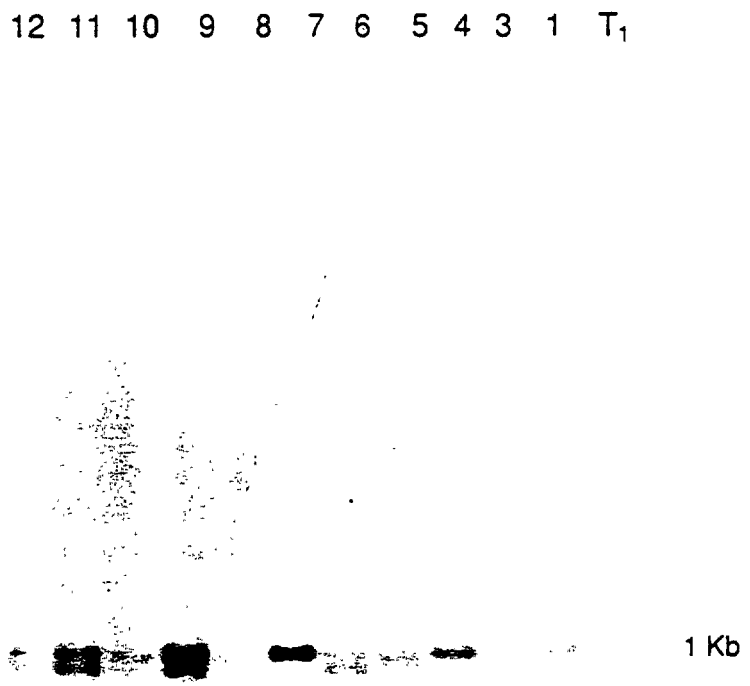
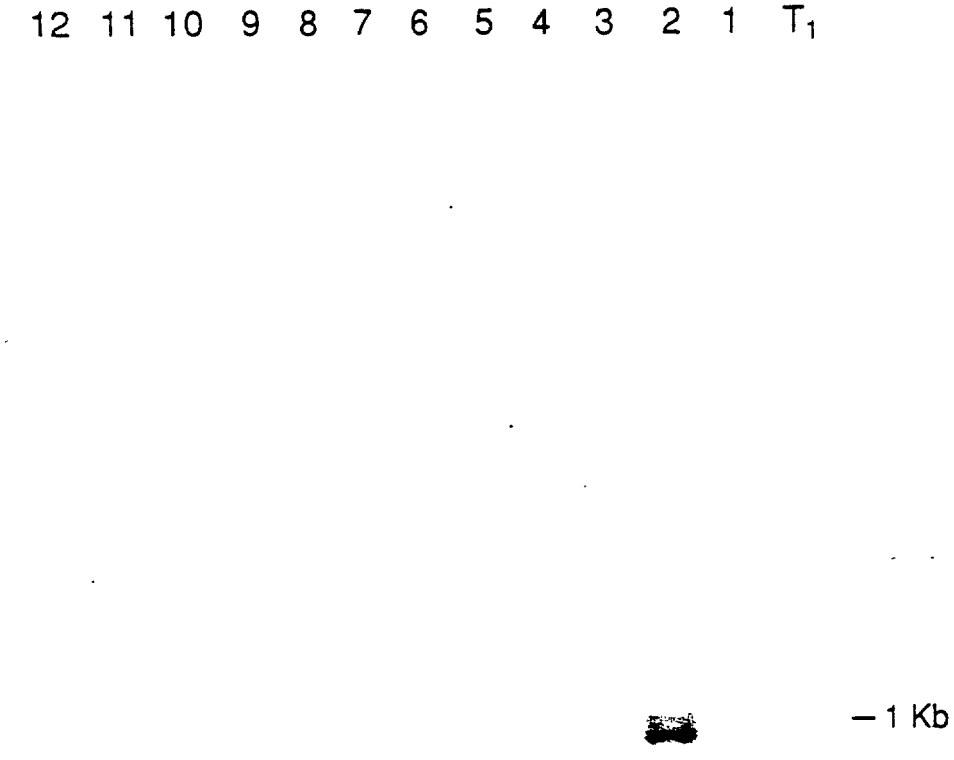


Figure 21. Southern blot of DNA from T_2 progeny of transgenic line 28B-425 digested with BamHI and BglII and hybridized with PG probe. DNA from the original T_1 28B-425 plant is shown in the far right lane. Individual T_2 progeny are designated by number.



line examined were all identified, with only one exception, by Southern analysis. In the case of line 7B-92, two of nine progeny examined were homozygous and seven heterozygous for the FLAVR SAVR™ gene. The fact that no homozygous null individuals were identified was probably due to the small number of individuals examined from the parent 7B-92 plant. The numbers of individual progeny identified in each of the three genetic classes were also consistent with the 1:2:1 ratio of homozygous positive, heterozygous and homozygous null individuals expected to result from self-fertilization of plants in which the T-DNA region(s), carrying the FLAVR SAVR gene(s) and the *kan*^{r} gene(s), has been inserted at only one physical site in the plant genome. Analysis of progeny, using the kanamycin germination assay (Appendix D-3), from those T_2 plants determined to be homozygous or heterozygous for the inserted DNA based on this Southern analysis agreed with these results (Table 1).

VIII. Summary.

An analysis of the number of FLAVR SAVR and *kan*^{r} genes and the number of genetic loci at which those genes are located has been presented for 8 independently derived transgenic tomato lines derived from 5 different cultivars. The data generated demonstrate the types of methods that can be used to determine the number of FLAVR SAVR and *kan*^{r} genes in any transformed tomato variety. The primary method devised to determine the number of FLAVR SAVR genes utilizes the endogenous PG gene, present in two copies per diploid tomato cell, as an internal gene copy number standard. Through use of this method it was determined that one copy of the FLAVR SAVR gene was present per diploid cell in the T_1 plant 501-1001, two copies of the FLAVR SAVR gene were present per diploid cell in six other original T_1 plants, and more than two copies of the FLAVR SAVR gene were present per diploid cell in the T_1 plant 501-1035 plant.

Several different lines of evidence were presented in support of the primary method used to determine the number of FLAVR SAVR genes. The number of borders between the T-DNA region(s) carrying the FLAVR SAVR gene(s) and the plant DNA into which the T-DNA had been inserted was determined. For simple insertions of single T-DNA regions into the tomato genome, the number of borders representing each end of a T-DNA region should be equal and represent the total number of T-DNA regions inserted into the tomato genome. The design of the border analyses presented here assumed that physical linkage between the right and left ends of the T-DNA region present in pCGN1436 was retained in transgenic plants. Evidence demonstrating physical linkage between the two genes in the transgenic plant lines examined here was presented (see Section V above).

Hybridization probes representing the two genes were then used to determine the number of right and left borders between the T-DNA region(s) carrying the two genes and the genomic DNA into which the T-DNA had been inserted. For the case of plant 501-1001, only one right border and one left border were detected. This data indicates that only one complete copy of the T-DNA region carrying both the FLAVR SAVR™ gene and the *kan*^{r} gene had been inserted into the genome of this plant. The presence of one FLAVR SAVR gene as determined by border analysis of plant 501-1001 was in agreement with the copy number of FLAVR SAVR genes derived for this plant through the use of the internal PG gene copy number standard.

While only one border involving the right end of the T-DNA was detected for all of the remaining seven transgenic lines used as examples for this analysis, two borders involving the left end of the T-DNA were evident for each. These data do not fit the case for insertion of two single T-DNA regions into independent loci in the tomato genome as two right borders as well as two left borders would be expected. The data are, however, consistent with the insertion of two T-DNA regions, in a head to head arrangement, into one locus. The insertion of *A. tumefaciens* T-DNA regions into plant genomes in these inverted repeat structures has been previously documented (Jorgensen *et al.* 1987). The molecular weight of the single DNA fragment hybridizing to the right border probe and the fact that the molecular weight of that fragment was the same in all seven transgenic lines supports this conclusion.

If the T-DNA regions carrying the FLAVR SAVR and *kan*^{r} genes did exist in inverted repeat structures in which the T-DNA regions were directly adjacent, then the molecular weights of various DNA fragments spanning the junction(s) could be predicted. Experiments testing these predictions were performed, and the results of the analyses were that all seven transgenic lines contained a DNA fragment that fit the predicted description of a junction fragment between two T-DNA regions adjacent at their right borders. An additional DNA fragment was detected in DNA isolated from plant 501-1035, consistent with an additional T-DNA region inserted into this plant's genome. This additional T-DNA region was not expected to exist at another locus in the genome based on the number of detected DNA border fragments. If the T-DNA regions in this plant all existed at one locus in head to head and tail to tail arrangement, the additional DNA fragment would represent a border between the right end of the T-DNA element in the structure not involved in a junction between T-DNA right ends, and plant DNA. Because this structure of three T-DNA regions oriented in inverted repeat units includes a junction between left ends of two T-DNA regions, Southern analysis designed to examine this junction was conducted. Evidence was provided not only for a junction between T-DNA region left ends in this plant but for its general structure of three T-DNA regions at one locus as well.

[redacted]

The structure of the T-DNA regions presented here in each case support the data on copy number of the FLAVR SAVR™ genes derived by comparison to the endogenous PG gene, and indicate that in the seven cases in which multiple copies of the FLAVR SAVR gene had been detected, all of the T-DNA regions containing those genes have been inserted at one physical locus in the genome. The presence of the T-DNA regions, containing the FLAVR SAVR genes and the physically linked *kan*^{r} genes, at one locus in each transgenic line is also supported by the data shown in Table 1. As judged by the ratio of resistant to susceptible T_2 progeny germinated on kanamycin, the *kan*^{r} gene (and therefore the physically linked FLAVR SAVR gene) of the T-DNA region behaves as a single Mendelian genetic trait. Southern analyses also demonstrated that DNA fragments, representing both the FLAVR SAVR gene and the *kan*^{r} genes, segregate among the progeny of the eight T_1 plants in a manner consistent with inheritance of these genes as a single genetic locus.

APPENDIX EA-5

Amino Acid Composition of APH(3')II

[redacted]

Amino acid composition of Aminoglycoside 3'-phosphotransferase II

Amino Acid	mol %	
	Derived from nucleotide sequence	Determined by amino acid analysis[a]
Ala	13.2	14.7
Arg	7.5	7.5
Asp	9.4	11.4
Asn	1.1	
Cys	1.9	0.0
Glu	6.8	12.0
Gln	4.2	
Gly	7.9	8.3
His	2.6	2.8
Ile	3.8	4.0
Leu	12.1	13.0
Lys	1.5	1.8
Met	2.3	2.3
Phe	4.2	4.4
Pro	4.2	5.2
Ser	3.8	3.5
Thr	3.8	3.6
Trp	1.9	nd[b]
Tyr	1.5	1.9
Val	6.0	3.6

[a] Beck et al. (1982)
[b] Not determined.

[redacted]

APPENDIX EA-6

APH(3')II, APH(3')II Antibody and Immunoblot

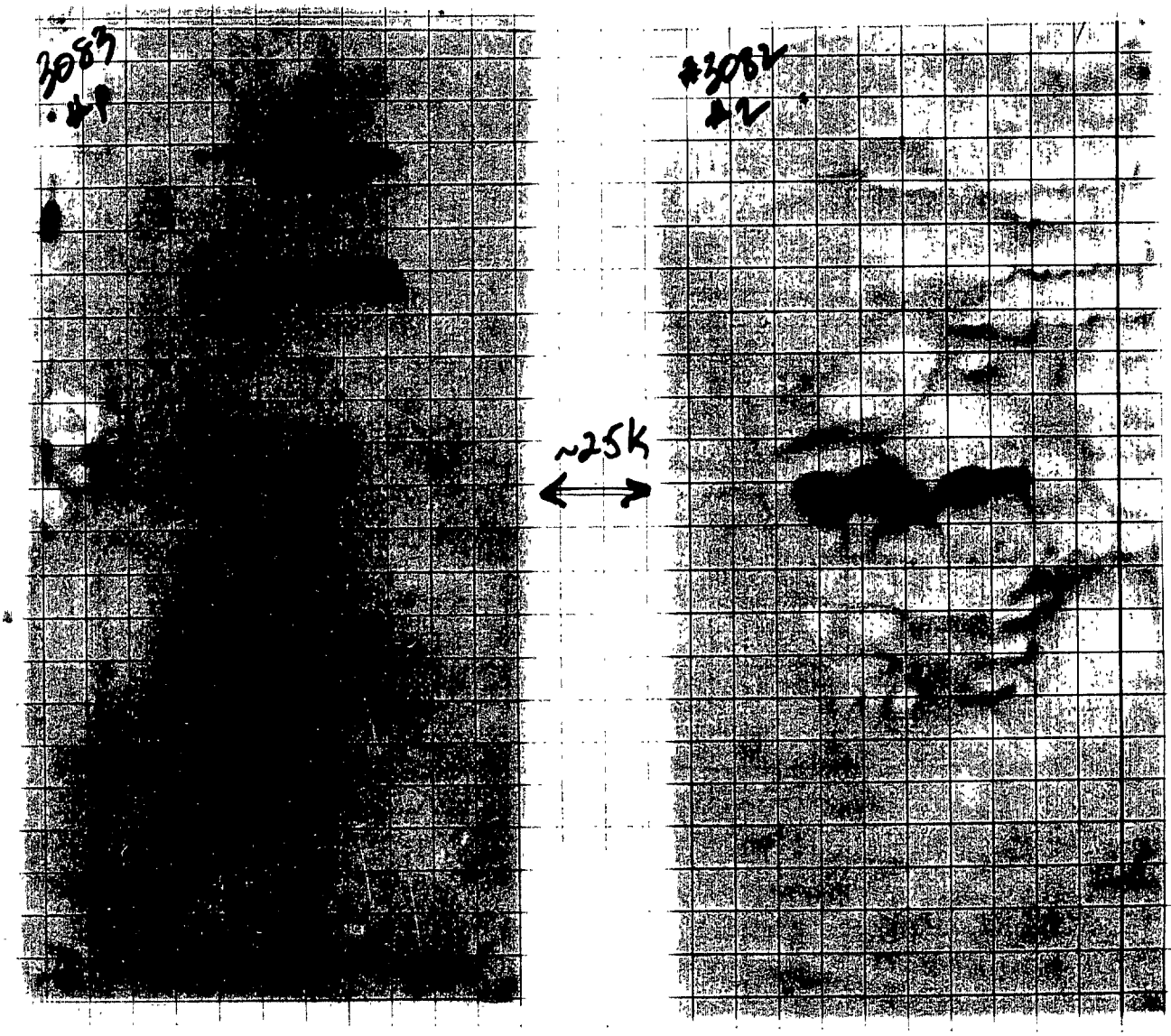
Description of characterization of the rabbit polyclonal antibody. Antibodies against APH(3')II were prepared by Antibodies Inc., Davis, CA by injection of polyacrylamide gel-purified APH(3')II into rabbits (pages 94-95, Redenbaugh et al. 1992). The resulting antiserum was first tested by Western blotting against extracts of *E. coli* SK1592/Tn*5* for specificity. As shown in Figure 1, antiserum 3082 reacted specifically with a single protein of molecular weight approximately 25,000 and was pursued further.

Following two booster injections of APH(3')II and subsequent bleedings, antiserum 3082-3 (third bleed) was tested against purified APH(3')II, as shown in Figure 2. An intense reaction resulted. This is the same antiserum used in D.4.3 (page 103, Redenbaugh et al. 1992).

The specificity of the APH(3')II antiserum is further shown in Figure 3. Soybean root nodule crude extracts nodulated with *Bradyrhizobium japonicum*/Tn5 were fractionated on a HPLC high resolution gel filtration column, and the eluted fractions assayed for APH(3')II activity. A single peak of activity was detected and appeared in fractions 34-45 (Figure 3A). Fractions 33-45 were subjected to Western blot analysis. Figure 3B demonstrates an immunoreactive polypeptide of correct molecular weight contained in HPLC fractions and in correspondence with the observed APH(3')II activity. The lack of reaction with other polypeptides, and the exact correlation between APH(3')II activity and protein, demonstrate the specificity of the anti-serum.

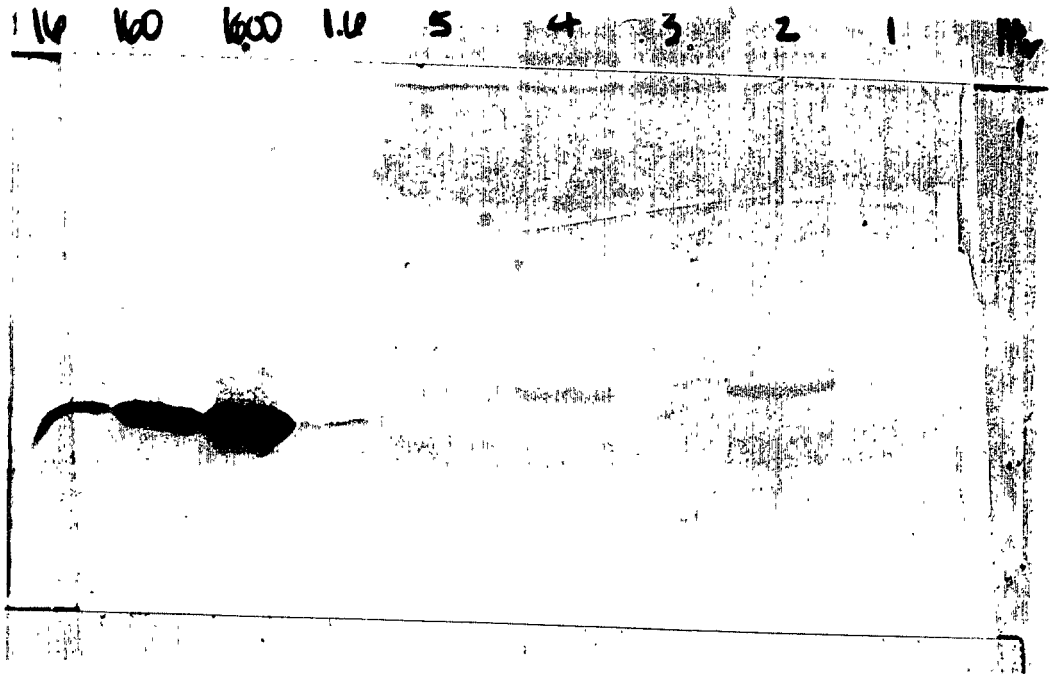
[redacted]

Figure 1. Reaction of APH(3')II antisera against E. coli/Tn*5* extract. Antisera was used at 1:50 dilution. Serum lots 3082 and 3083 represent the initial bleeds of two different rabbits.



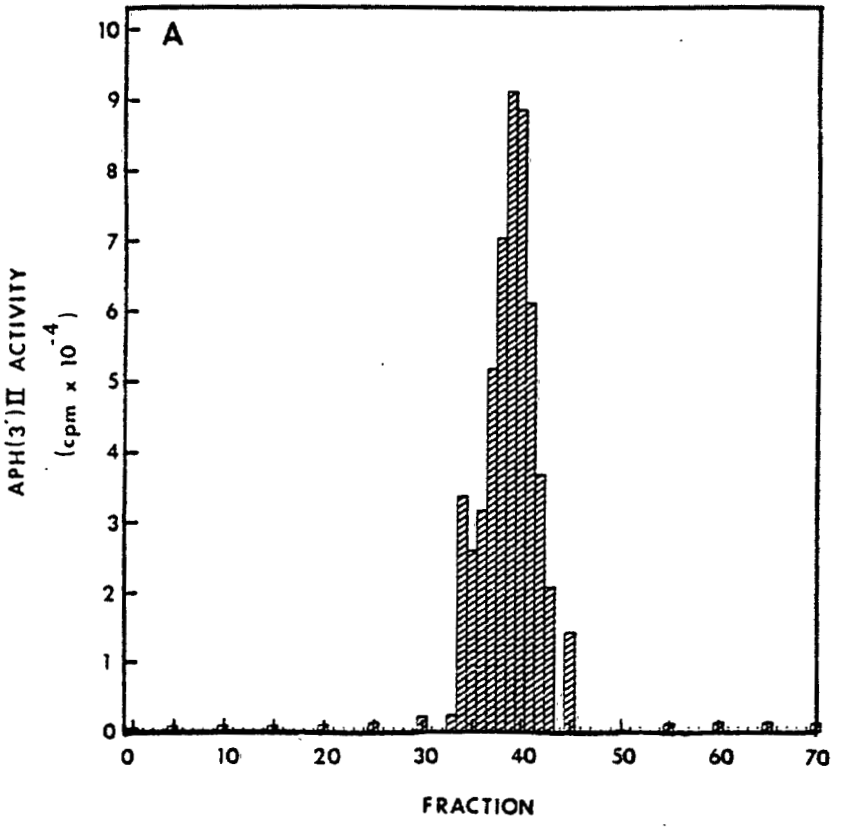
[redacted]

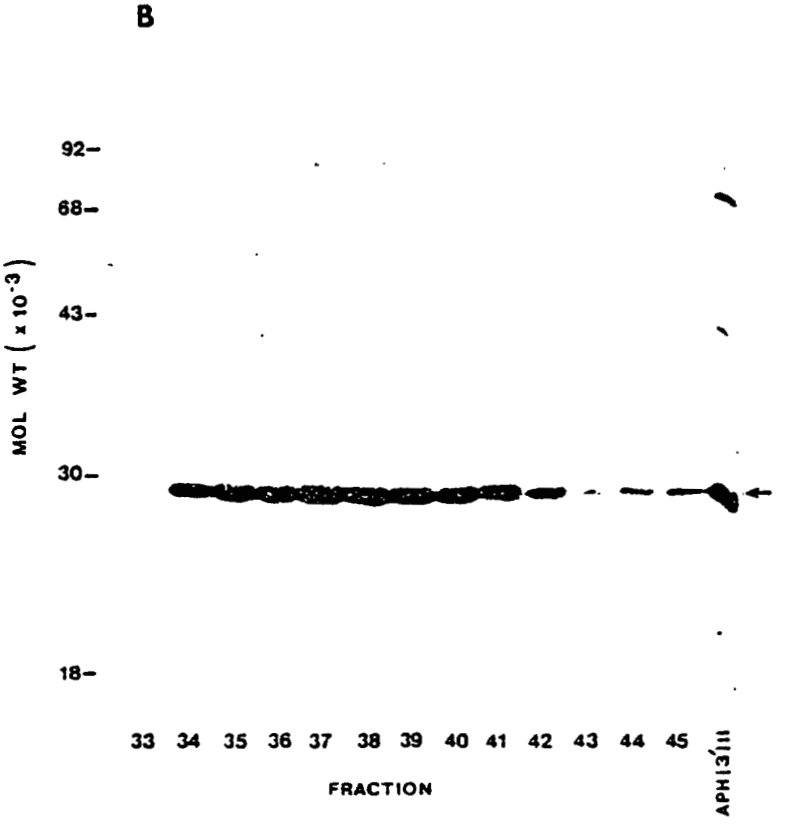
Figure 2. Reaction of APH(3')II antisera against purified APH(3')II. Purified protein from 5' Prime-3' Prime was present at 16, 160 and 1600 ng. Antiserum was used at a 1:50 dilution.



[redacted]

Figure 3. High resolution HPLC and western blot analysis of APH(3')II in soybean root nodules. (A) APH(3')II activity pattern of fractions obtained after soybean nodule extracts were fractionated by HPLC high resolution gel filtration chromatography. (B) Western blot analysis of immunoreactive polypeptides contained by HPLC fractions. Anti-APH(3')II antisera was used to complex the APH(3')II protein, and the complex visualized with ^{125}I-labeled protein A. The arrow designates the position of authentic APH(3')II purified from *E. coli*::Tn5.





[redacted]

Information on the purity of the APH(3')II protein that was used as a source of APH(3')II in the immunoblot. Purified Tn*5*-encoded APH(3')II protein was obtained from 5 Prime -> 3 Prime, Inc.® (current address 5603 Arapahoe, Boulder, CO). As per the attached product specification sheet (Neomycin Phosphotransferase II), the protein has a >98% purity, determined by denaturing SDS polyacrylamide gel electrophoresis.

[redacted]

NEOMYCIN PHOSPHOTRANSFERASE II (NPT II)

FOR RESEARCH USE ONLY

Product	Catalog No.	Size
Neomycin Phosphotransferase II (NPT II)	5306-639134	50 µg

Lot Number: CD212A
Protein Concentration: 0.75 mg/ml

GENERAL

The NPT II enzyme has been affinity purified from an *E. coli* strain which contains the NPT II gene isolated from transposon Tn5. The NPT II protein migrates at approximately 29,000 daltons during denaturing SDS-Polyacrylamide gel electrophoresis.

COMPOSITION

The preparation is supplied in 50 mM Tris-Cl, pH 7.5, 200 mM NaCl, 3 mM KCl, 1 mM β-mercaptoethanol, 10 µg/ml Neomycin, and 50% glycerol.

STORAGE

Store at -20°C in a non-frost-free freezer. Avoid repeated freeze-thaw cycles.

STABILITY

Stable for up to 12 months when the stock solution is stored as described. NPT II is unstable in dilute solution.

CONCENTRATION

The protein concentration was determined by Bradford protein assay using a BSA standard curve.

PURITY

≥ 98% as determined by denaturing SDS polyacrylamide gel electrophoresis.

ENZYME ACTIVITY

One unit of NPT II will convert 1.0 nmole of ATP and kanamycin to ADP and phosphorylated kanamycin per minute at 37°C.

Enzyme activity may be determined by the following modification of the method described by Franke and Hruby[1].

1. Prepare the following reaction:

10µl Assay buffer	67 mM Tris maleate, pH 7.1, 40 mM $MgCl_2$, 400 mM NH_4Cl, 1.67 mM Dithiothreitol
10 µl [γ-^{32}P] ATP (34 pmole)	
10 µl NPT II (~ 1 ng)	
2 µl 1 mg/ml Kanamycin	

2. Mix and incubate the reaction for 15 minutes at 37°C.

3. Spot onto 1 cm^2 Whatman P81 paper.

4. Wash P81 paper 5 minutes in H_2O at 90°C.

5. Wash P81 paper 3 times with H_2O at 22°C.

6. Dry P81 paper and count the bound phosphorylated kanamycin in 5 ml of scintillation fluid.

REFERENCE

1. Franke, C.A. and D.E. Hruby. 1987. J. Virol. Meth. 16:147-154.

07/92
NPTII

5 Prime → 3 Prime, Inc.®
5603 Arapahoe
Boulder, CO 80303 USA
800-533-5703 (U.S. & Canada)
FAX: 303-440-0835
LOCAL: 303-440-3705

Reaction conditions for immunoblot and explanation of nonspecific bands.

The reaction conditions were modified from the ProtoBlot™ Immunoscreening System Promega Biotec Technical Manual (see attached) and are outlined in the attached protocol (see Immunoblot 1).

The presence of nonspecific bands that appear in the lanes containing the control with 160 ng APH(3')II and transformed tomato extracts is characteristic of many immunoblots. According to Harlow and Lane (1988 Antibodies - A Laboratory Manual; see attached), "the most frequently encountered problem in immunoblots is the presence of a high background consisting either of extra, discrete bands or of a general diffuse signal covering the entire membrane." Because of the low concentration of APH(3')II in transgenic plants, the antiserum was used at a low dilution with the blot and under conditions (type of blocking buffer, extended time) which maximize binding of the antibody. These conditions result in a high background level due to non-specific interactions. Finally, these bands are present in both control and transgenic plants and are of molecular weights that do not interfere with measurement of the APH(3')II protein.

[redacted]

ProtoBlot™ Immunoscreening System

Promega Biotec

Technical Manual

2800 S. Fish Hatchery Road	
Madison WI 53711 USA	
Toll Free	800-356-9526
Telephone	608 274-4330
TWX/Telex	910-286-2738

[redacted]

with the surface that was in contact with the agar facing up.

2. To saturate nonspecific protein binding sites, incubate the filters in TBST + 20% calf serum or other suitable protein blocking agent (e.g., 1% BSA, 1% gelatin, etc.) for 15 to 30 minutes. Use 7.5ml per 82mm filter and 15ml per 132mm filter.

6. Bind primary antibody. Incubate the filters in TBST + primary antibody for 30 minutes. Use 7.5ml per 82mm filter and 15ml per 132mm filter. Use serum antibody or ascites fluid at 1:200 - 1:1000 dilution. The positive control mouse anti-β-galactosidase antibody can be used at 1:5000. The diluted primary antibody can be reused several times.

NOTES:
1. The quality of the antibody probe is clearly important. High titer, high affinity antibodies produce better signals than low titer, low affinity antibodies. In general, antibodies that produce good signals on Western blots will produce good signals in the screening procedure at similar dilutions. If little or no signal is obtained, or if the background is too high in the screening procedure, the antiserum or ascites should be checked on Western or "dot" blots before proceeding. (The **ProtoBlot** system anti-rabbit and/or anti-mouse antibodies and substrates can be used as described

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here for these types of blotting.) A few antibodies, particularly some monoclonals, are conformation-dependent and do not recognize antigens immobilized on a surface. Others may tend to stick non-specifically to surfaces such as nitrocellulose. These antibodies are not suitable for immunoscreening in this manner.

2. Most primary antibody preparations can be used in the 1:1000 dilution range. Unnecessarily high concentrations of some antisera can accentuate backgrounds produced by anti-*E. coli* antibodies which may be present at low titers. (See Section IV on the use of the *E. coli* extract for background reduction.)

7. Wash. Wash the filters in 15 - 20ml TBST three times for 5 to 10 minutes each.

8. Bind anti-rabbit or anti-mouse IgG alkaline phosphatase conjugate. Transfer the filters to TBST containing the appropriate second antibody - alkaline phosphatase conjugate (1:7500 dilution is recommended). Allow 7.5ml per 82mm filter and 15ml per 132mm filter. Incubate for 30 minutes at room temperature.

9. Wash. Wash the filters in TBST, as in step 7.

10. Start color reaction. Blot the filters damp dry on filter paper

and transfer to the color development substrate solution, prepared as follows: for 5ml solution, add 33µl NBT substrate to 5ml AP buffer (100mM Tris-HCl, pH 9.5, 100mM NaCl, 5mM $MgCl_2$), mix, add 16.5µl BCIP substrate and mix again. Protect the solution from strong light and use within 1 hour. Positive clones will appear as purple plaques on the filters. Development of color will continue for at least 4 hours, although filters left overnight tend to have high backgrounds.

NOTE:
1. If either of the substrates has precipitated during storage, warm the vial to room temperature and mix to redissolve before use.

11. Stop color development. When the color has developed to the desired intensity, stop the reaction by replacing the substrate solution with stop solution (20mM Tris-HCl, pH 8.0, 5mM EDTA). The filters can be stored in this solution or dried. The color will fade slightly upon drying but can be restored by moistening the filter with water.

12. Plaque purification. To retest a putative positive signal, remove an agar plug containing phage particles from the region of the plate corresponding to the signal on the filter. Incubate the agar plug in 1ml of λ diluent for at least 1 hour at room temperature, vortexing occasionally. Replate

IMMUNOBLOT 1

TBST
20 ml 1M Tris pH8.0
100ml 5M NaCl
1ml Tween 20
Final volume 2000L

Electroblot Buffer
Tris base 18.2 g
Glycine 86.5 g
Methanol 1200 ml
Bring to 6000 ml w/ DDi

Substrate Buffer
10 ml ~~1~~ Tris pH 9.5 1M
2ml 5M NaCl
5ml 1M MgCl2
Final volume 100ml

1. Soaked gel, nitrocellulose membrane, 3MM paper, and support pads in Electro blot buffer (nitrocellulose was soaked briefly in H20 first).

The plastic screens were clamped closed around the gel sandwich and the whole unit was placed in the electroblot tank with the nitrocellulose membrane closest to the positive electrode.

2. Fill the unit with electroblot buffer and run blot at full voltage 1-2 hrs. until needle reads 1.

3. Dismantle gel sandwich and place nitrocellulose in a glass pan and quickly rinse with water.

4. Briefly rinse in TBST.

[redacted]

5. Blot 50 ml 1% BSA and TBST (0.5 g BSA in 50 ml TBST) for 30 minutes.

6. Rinse with TBST.

7. Put in APH(3')II 3082-3 diluted 1:100 in TBST for 30 minutes.

8. Rinse with TBST for 5 minutes 3 times.

9. Put in secondary antibody 1:5000 dilution for 30 minutes
   6 µL goat anti-rabbit
   50 ml TBST

10. Wash 3 times in TBST for 5 minutes.

11. Final rinse in substrate buffer.

12. Stain
    44 µl BCIP
    33 µl NBT
    10 mls substrate buffer

13. Shake until developed

14. Dry nitrocellulose on paper and wrap in saran wrap.

[redacted]

Antibodies

A LABORATORY MANUAL

Ed Harlow
Cold Spring Harbor Laboratory

David Lane
Imperial Cancer Research Fund Laboratories

Cold Spring Harbor Laboratory
1988

Antibodies
A LABORATORY MANUAL

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All Cold Spring Harbor Laboratory publications may be ordered directly from Cold Spring Harbor Laboratory, Box 100, Cold Spring Harbor, New York 11724. Phone: 1-800-843-4388. In New York (516) 367-8423.

COMMENTS ■ Troubleshooting—Bad Backgrounds

The most frequently encountered problem in immunoblots is the presence of a high background consisting either of extra, discrete bands or of a general diffuse signal covering the entire membrane.

Specific background bands are generated either by contaminating antibodies present in polyclonal sera or by specific cross-reactions between different antigens. The latter problem is especially prevalent with monoclonal antibodies. Nonspecific diffuse backgrounds result either from insufficient blocking of the membrane or from a specific reaction of the detecting reagent with a component of the blocking buffer.

Diffuse Backgrounds

- If using an indirect technique, first determine whether the secondary reagent is contributing to the background. Omit the primary antibody and observe the background generated by the secondary reagent alone. If the background is generated by the secondary reagent: (1) use an alternative label; (2) try adsorbing the secondary reagent with a sample of the starting antigen preparation (an acetone powder may be suitable); (3) reduce the time in substrate or use a less sensitive substrate.
- Use a different blocking buffer. Try Blotto/Tween, if presently using anything else.
- Titrate the concentration of the primary and secondary antibodies.
- Reduce the incubation time of the primary and secondary antibodies.
- Wash the filter at each step in 1% NP-40, 0.5% DOC, 0.1% SDS, 150 mM NaCl, 50 mM Tris (pH 7.5).
- Add 1% NP-40 to the primary and secondary antibodies.
- Increase the duration of each washing step.

Specific Background Bands

- If using an indirect technique, first determine whether the secondary detecting reagent is contributing to the background. Omit the primary antibody and observe the background generated by the secondary reagent alone. If the background bands are generated by the secondary reagent, first use an alternative label. Second, try adsorbing the secondary reagent with a sample of the starting antigen preparation. An acetone powder may be suitable.
- If using a monoclonal antibody, use another monoclonal antibody if available. Continued presence of the same band may suggest an amino acid homology between your antigen and the other band.
- If using a polyclonal serum as a source of primary antibodies, try adsorbing the serum with a protein preparation that does not contain the antigen of interest.

APPENDIX EA-7

Calgene Laboratory and Greenhouse Containment Procedures Implemented by Calgene to Comply with NIH, USDA and EPA Guidelines

[redacted]

LABORATORY PROCEDURES

The research at Calgene falls under the guidelines for a BL1 Lab according to NIH Guidelines.

GUIDELINES

1). Access to the laboratory is limited or restricted at the discretion of the Health & Safety Officer when experiments are in progress.

2). Work surfaces are decontaminated once a day and immediately after any spill of viable rDNA material. (Decontamination can be done using a 2.5% Bleach solution)

3). All contaminated laboratory wastes must be decontaminated prior to disposal. (Kill bins are located throughout the labs for autoclaving)

4). Mechanical pipetting devices shall be used; mouth pipetting is prohibited. (Mechanical pipettes available in all labs)

5). Eating, drinking, smoking and applying cosmetics are not permitted in the work area. Food may only be stored in refrigerators or stored in cabinets outside of the lab areas.

6). Persons shall wash their hands after handling organisms containing recombinant DNA molecules and before they leave the laboratory.

7). All procedures with rDNA are performed carefully to minimize the creation of aerosols (e.g., manipulations such as inserting a hot inoculating loop or needle into a culture, or forceful ejection of fluids from pipettes or syringes)

8). It is strongly recommended that laboratory coats, gowns, or uniforms be worn to prevent contamination or soiling of street clothes. (Clean lab coats are available in the locker outside 1910 main. Lab coats are to be worn in lab areas only.

Special Containment Practices

1). Contaminated materials that are to be steam sterilized (autoclaved) or decontaminated at a site away from the laboratory shall be placed in a durable leakproof container which is closed before being removed from the laboratory.

2). Special containment equipment is generally not required for manipulations of agents assigned to the Biosafety Level 1 category.

[redacted]

rDNA First-Aid Procedure

1). In case of *skin* contact with rDNA, wash the area thoroughly with soap and water.

2). Flush the *eyes* with copious amounts of water for at least 15 minutes.

Spill Containment Procedure

1). Immediately alert personnel in the area of the spill and cordon off the area with tape or barricades.

2). Contain the spill from entering floor drains with either solid absorbent or paper towels.

3). Contact the Health and Safety Department for additional decontamination assistance.

4). Wear the appropriate personal protective equipment, (e.g., lab coat, latex gloves, safety glasses)

5). Pour 2.5% bleach directly on the spill area to kill any biological material. Wait at least 5 minutes before proceeding to the next step.

6). Clean up the spill with paper towels and dispose in regular trash unless toxic components still exist in decontaminated culture medium. Call the Health and Safety department for disposal collection.

[redacted]

BL1 CONTAINMENT GUIDELINES

Calgene
Planting Operations
Revised 10/92

POLICY

The BL1 containment policy premise is that all transgenic plant material shall be grown, transported, tested, stored and disposed of under conditions which shall minimize the probability of gene escape. Procedures will be clear, well documented and consistent among work areas. BL1 material shall be clearly labeled. Accurate records shall be kept. Personnel with access to BL1 material will be trained in all procedures necessary for the performance of their work.

SIGNS

1. All greenhouses holding BL1 material are clearly labeled with the "Genetically Engineered Plant Material In This Area" sign. Employees are informed at orientation of the relationship between this sign and transformed plants.
2. All BL1 plants are labeled with an orange label.
3. Seed packets containing seed from BL1 plants are stamped "BL1 REGULATED" in orange red ink on the envelope.

RECORDS

Each plant receives a unique planting request number. Records are kept giving information such as plant type, variety, construction, planting date, location, responsible scientist and trash date.

GREENHOUSE

1. Sinks are located in each greenhouse. All workers are required to wash hands upon entering and before leaving each house, thus reducing chances of accidental transfer of BL1 tissue.
2. Greenhouse interiors are kept weed-free.
3. Labeled receptacles for discarded BL1 plant material and weeds are in each house.
4. The structure is inspected monthly for holes where pollinating insects and rodents could enter or escape.
5. An Integrated Pest Management program is in place to control insects and rodents, including weekly pest monitoring.
6. All doors are equipped with locks.

SURROUNDING AREA

1. Weeds are kept under control around the perimeter to reduce possible pollen

[redacted]

acceptors and donors, as well as reduce pest habitats.

2. Yearly weed surveys are taken of the surrounding greenhouse property flora. Possible escapes are monitored and destroyed as well as possible pollen acceptors and donators.

MOVEMENT OF PLANT MATERIAL BETWEEN FACILITIES

1. BL1 tissue is transported in enclosed authorized Calgene vehicles only.
2. After transport, the vehicle interior is inspected for any fallen plant parts. All tissues must undergo designated disposal procedures.
3. Moving plants between greenhouses within a site will be done as quickly as possible. After movement is complete, check the travel path for fallen plant parts.
4. Tissue samples taken from one site to another shall be labeled and transported in an enclosed vehicle.
5. Field trial transplants may be hardened off outside within a secured area, if no reproductive organs have developed.

DESIGNATED DISPOSAL PROCEDURE

1. All plant material (including fruit, stems, leaves, tubers and roots) and used soil is disposed of by one of the following methods to insure that no genetically engineered material survives: 1) steam pasteurization, 2) autoclaving, or 3) fumigation, using registered soil fumigants such as methyl bromide or sodium methyldithiocarbamate (Vapam or Metam). For #1 and #2, the length of treatment time is determined by seed kill testing prior to disposal. For #3, standard fumigation practices will be used. The procedure consists of spreading plant material and soil on the ground outside the greenhouses, covering the material with a tarp, treating it with the fumigant, incorporating it into ground and subsequently checking for volunteer plants.
2. Crop seed samples are placed periodically in the bottom, middle and top of the soil/plant material to test the killing-thoroughness of treatment. Germination tests are run. A non-treated control is included to show the germination rate of the original seed.
3. All non-transformed plants grown in the same house as transformed plants of the same crop are treated as BL1 plants when reproductive organs and seeds have developed.
4. Disposal material from off site facilities will be transported in an authorized enclosed vehicle. Vegetative material will be placed in plastic bags. Soil may be transported in bags or pots in an enclosed vehicle.
5. Material awaiting disposal at off site facilities shall be stored in an enclosed area i.e., greenhouse or headhouse. Material shall not be stored outside.

POLLEN MOVEMENT

Installing physical barriers to prevent wind-borne pollen movement and dispersal

[redacted]

could seriously impair the effectiveness of the greenhouse cooling system. Physical barriers are in place to prevent insect movement into the greenhouses.

[redacted]